Veterinary Microbiology 182 (2016) 28–34

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Emergence of a sylvatic enzootic formosan ferret badger-associated rabies in Taiwan and the geographical separation of two phylogenetic groups of rabies viruses

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ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 17 September 2015
Accepted 28 October 2015

Keywords:
Rabies
Formosan ferret badger
Sylvatic
Phylogenetic analysis
Nucleoprotein
Glycoprotein

ABSTRACT

Taiwan had been declared rabies-free in humans and domestic animals for five decades until July 2013, when surprisingly, three Formosan ferret badgers (FB) were diagnosed with rabies. Since then, a variety of wild carnivores and other wildlife species have been found dead, neurologically ill, or exhibiting aggressive behaviors around the island. To determine the affected animal species, geographic areas, and environments, animal bodies were examined for rabies by direct fluorescent antibody test (FAT). The viral genomes from the brains of selected rabid animals were sequenced for the phylogeny of rabies viruses (RABV). Out of a total of 1016 wild carnivores, 276/831 (33.2%) Formosan FBs were FAT positive, with occasional biting incidents in 1 dog and suspected spillover in 1 house shrew. All other animals tested, including dogs, cats, bats, mice, house shrews, and squirrels, were rabies-negative. The rabies was badger-associated and confined to nine counties/cities in sylvatic environments. Phylogeny of nucleoprotein and glycoprotein genes from 59 Formosan FB-associated RABV revealed them to be clustered in two distinct groups, TWI and TWII, consistent with the geographic segregation into western and eastern Taiwan provided by the Central Mountain Range and into northern rabies-free and central-southern rabies-affected regions by a river bisecting western Taiwan. The unique features of geographic and genetic segregation, sylvatic enzooticity, and FB-association of RABV suggest a logical strategy for the control of rabies in this nation.

1. Introduction

Rabies can affect a wide range of mammals, and exposure to rabies virus (RABV) without timely treatment invariably causes fatal neurological diseases. Rabies is distributed throughout most of the world and causes more than 55,000 human deaths annually, of which >50% occur in Asia and Africa, where rabid dogs serve as the major source of infection (Knobel et al., 2005).

The rabies virus is bullet-shaped and has a single-stranded negative-sense RNA genome. The RABV genome contains five genes, denoted N, P, M, G, and L, encoding nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase, respectively. The G protein, which produces spike-like projections on the viral envelope for reacting with cellular receptors, is important in determining host range. The N protein is involved in the regulation of genome replication and transcription (Wunner and Conzelmann, 2013). The N and G genes are the most extensively studied in RABV and are usually subjected to phylogenetic analysis.

Rabies epidemics had been documented in Taiwan since 1947. The epidemics were gradually controlled through massive vaccination of dogs and control of dog populations. By 1959, no human cases were reported, and by 1961, no animal cases (Liu, 2013). The five decades of the apparent rabies-free status in animals changed when 3 wild Formosan ferret badgers (Formosan FBs, *Melogale moschata subaurantiaca*) were diagnosed with rabies in July 2013 (OIE, 2013). Later in 2013, an increasing number of dead, ill, or euthanized Formosan FBs and other wild animals were

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http://dx.doi.org/10.1016/j.vetmic.2015.10.030
0378-1135/

submitted to the Animal Health Research Institute (AHRI), Council of Agriculture, Executive Yuan, Taiwan, for rabies diagnosis.

Following the surprising epidemic, questions arose as to where the RABV came from, what the circulating RABV was, what animal species were affected, and where the affected animals were distributed. A recent study suggests that the most recent common ancestor of Formosan FB-associated RABVs in Taiwan originated 91–113 years ago (Chiou et al., 2014). However, the spectrum of host species and the geographic distribution of affected animals have remained unclear.

In the present study, a large number of clinical samples and background information were compiled to answer the aforementioned questions. The full-length sequences of the N and G genes of 59 RABVs from brains of selected rabid Formosan FBs were subjected to phylogenetic analysis.

2. Materials and methods

2.1. Animal specimens

Through the cooperation of local animal disease control Centers, forestry workers, wildlife rescue and rehabilitation stations, wild carnivores and other wildlife species that were found dead or ill with neurologic signs, especially those exhibiting aggressive behaviors or having bitten or scratched people, were submitted to the AHRI for rabies diagnosis. The submitted animals were necropsied in a negative pressure facility to obtain brain tissues.

2.2. Fluorescent antibody test

Brain tissues in poor condition, such as decomposition, dehydration, and leakage from a broken skull, were excluded from RABV detection. The FAT was used to detect the presence of RABV antigen within brain tissues, based on the protocol developed by the Centers for Disease Control and Prevention, Atlanta, Georgia, USA (CDC website).

2.3. RNA extraction

The brains of selected FAT-positive Formosan FBs were subjected to RNA extraction (Tables 1 and 2). The brain tissues were emulsified with Minimal Essential Medium Eagle supplemented with Earle's Salt and L-glutamine (Mediatech, Manassas, Virginia, USA) to prepare a 10% w/v suspension. Total RNA was extracted using TRIzol (Life Technologies, Grand Island, New York, USA) following the manufacturer's instructions. The RNA was subjected to reverse transcription polymerase chain reaction (RT-PCR) to amplify the full-length N and G genes of RABV.

2.4. RT-PCR and nucleotide sequencing

According to the aligned sequences composing whole genome sequences of 10 FB RABVs provided by Dr. Chun-Hsien Tseng, two primer pairs were designed to amplify full-length of N gene and G gene. The 10 FB RABVs (their background information were shown in Tables 1 and 2) were detected in 2013 epidemic and their whole genome sequences were determined by Dr. Tseng and announced in AHRI website (AHRI website). The positions of primers were referred to the consensus sequence of the aligned sequences of whole genomes of 10 FB RABVs.

NF1 (21–43): 5′-AGAAGAAGCAGACAATGTCATCT-3′;
NR3 (1552–1531): 5′-GCTCTGATTGCACTCGGATTGA-3′;
GF1 (3284–3268): 5′-ACCTTTACATTTTAAGCCTCG-3′;
GR4 (4994–4974): 5′-CTGTTGCGAGGAGTCTAAAGA-3′.

Table 1
Rabies viruses of the Formosan ferret badger used for phylogenetic analyses.

Virus strain	Region of collection (county)	Genebank accession number	
		N gene	G gene
TW1680[a]	Taichung	KF501181	KF501175
Th1749	Taichung	KP860163	KP860212
TW1907[a]	Taichung	KP881355	KP881359
Th2170	Taichung	KP860164	KP860213
Th2229	Taichung	KP860165	KP860214
Th2284	Taichung	KP860166	KP860215
Th2408	Taichung	KP860167	KP860216
Th2484	Taichung	KP860168	KP860217
Th4957	Taichung	KP860169	KP860218
Th5114	Taichung	KP860170	KP860219
TW1682[a]	Nantou	KF501182	KF501176
TW1683[a]	Nantou	KF501183	KF501177
Nt1938	Nantou	KP860149	KP860198
Nt1983	Nantou	KP860150	KP860199
Nt2169	Nantou	KP860151	KP860200
Nt2269	Nantou	KP860152	KP860201
Nt2270	Nantou	KP860153	KP860202
Nt2274	Nantou	KP860154	KP860203
TW2700[a]	Nantou	KP881354	KP881358
Nt2702[b]	Nantou	KP860155	KP860204
Nt2704[b]	Nantou	KP860156	KP860205
Nt2706[b]	Nantou	KP860157	KP860206
Nt2710[b]	Nantou	KP860158	KP860207
Nt4951	Nantou	KP860159	KP860208
TW1944[a]	Yunlin	KP881356	KP881360
YL2167	Yunlin	KP860184	KP860233
YL5154	Yunlin	KP860185	KP860234
Cy2143	Chiayi	KP860137	KP860186
Cy2263	Chiayi	KP860138	KP860187
Cy2717[b]	Chiayi	KP860139	KP860188

[a] The 10 samples were detected in 2013 epidemic and their whole genome sequences were determined by Dr. Chun-Hsien Tseng and announced in AHRI website.
[b] Frozen archived samples. Nt2702 and Nt2704 were collected in 2010, Nt2706 in 2012, Nt2710 and Nt2717 in 2013.

The primers NF1 and NR3 were used for N gene amplification and primers GF1 and GR4 for G gene amplification. The RT-PCR was carried out in a final volume of 50 μl containing 20 pmole of forward and reverse primers, 25 nmole of each nucleotide triphosphate (TaKaRa Bio Inc., Dalian, China), 2 U of AMV reverse transcriptase (Promega, Madison, Wisconsin, USA), 20 U of ribonuclease inhibitor RNasin (Promega), 1.25 U of PrimeSTAR GXL DNA polymease (TaKaRa Bio), and 50 nmole of magnesium ion. The mixture was incubated at 42 °C for 40 min and 98 °C for 120 s, and then subjected to 30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 120 s, with a final extension at 68 °C for 7 min in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). After the RT-PCR, 8 μl of the product was electrophoresed and visualized, and amplicons with the expected molecular weights were sequenced by commercial services (Mission Biotech, Taipei, Taiwan). The amplicons were sequenced in an ABI 3730xl DNA analyzer using BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Each sample was sequenced at least twice.

2.5. Phylogenetic analysis

The geographical distribution of rabid Formosan FBs was plotted on a map of Taiwan (Fig. 1) using QGIS 2.4 software (Open Source Geospatial Foundation; Beaverton, Oregon, USA). To investigate the phylogenetic relationship between the Taiwanese RABVs, full-length nucleotide sequences of 59 N and 59 G genes of RABVs were used. Ten sequences of N gene and G gene were extracted from whole genome sequences of 10 FB RABVs provided by Dr. Chun-Hsien Tseng (the information of 10 FB RABVs was described in Section 2.4), and 49 were determined by the authors.