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A Case Report of Serological Evidence of Paramyxoviruses Related to *Porcine Orthorubulavirus* in Mexican Bats

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Abstract

In this report, we showed the presence of antibodies to *Porcine orthorubulavirus* (PRV) in Mexican bats using a serological approach. A total of 42 bats, belonging to seven different species, were sampled from two different refuges/caves, located near to a pig fattening area where spontaneous outbreaks of PRV had occurred. Analysis by serum-virus neutralizing and immunoperoxidase monolayer assay revealed the presence of antibodies in fifteen out of 42 investigated bats (i.e. 35%), six of them were also positive by *Paramyxoviridae* family using PCR assay targeting the L gene of paramyxoviruses. This case demonstrates for the first time antibodies detection of this virus in different bats species which is important for our understanding of PRV ecology, evolution and mechanism of cross-species transmission. These findings support the hypothesis that bats could act as an intermediate or natural host for interspecies transmission of certain paramyxoviruses.

Keywords: *Porcine orthorubulavirus*; Bats; Epidemiology; Inter-species transmission

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Introduction

Over the past years, a number of zoonotic and vector-borne viral diseases have emerged in Southeast Asia and the Western Pacific, with fruit bats as a wildlife reservoir. Bats have been implicated in numerous new emerging infectious diseases, through its role as reservoirs for viruses with the ability to occasionally cross species barriers [1]. One family of viruses with a particularly strong link to bats is *Paramyxoviridae* [2]. Bats seem to be an ancient reservoir of many paramyxovirus taxa [3], and it is possible to find a variety of paramyxovirus lineages in most bat families. In America, retrospective analyses have shown the presence of two different paramyxoviruses from the genus *orthorubulavirus*. One of them is *Mapuera Virus* (MapV) found in bats and the other is *Porcine orthorubulavirus* (PRV) found in pigs [4]. Recently PRV was renamed *Porcine orthorubulavirus*-La Piedad Michoacán Mexico Virus (LPMV-PRV) [5]. This virus was actually known as La Piedad, Michoacán Virus (LPMV) and emerged spontaneously in pigs in Mexico in the early 1980s [6,7]. Veterinarians reported an outbreak of a new type of encephalitis (named blue eye disease, BED) among piglets on swine farms around the city of La Piedad, in the Mexican state of Michoacán. The clinical signs were characterized by neurological disorders in newborn piglets [7] and neurological and respiratory disease in suckling and growing pigs [8], with or without corneal opacity [7]. In adult pigs, clinical signs are mainly associated with reproductive failure [9-11]. BED remains endemic in central and western-central parts of Mexico where there is a dense population of pigs. The disease has been serologically diagnosed in at least 16 states of Mexico, but has not yet been reported in other countries [12,13]. Since the first recognized outbreak in 1980, sporadic outbreaks of BED have continued to occur and the specific source of many of these outbreaks remains unknown. Genetic analysis has led to that PRV is classified within the genus *orthorubulavirus* and shows closest genetic relationship to the *Mapuera virus* (MapV, genus *Rubulavirus*) isolated from bats [4]. As fruit bats are considered to be the natural host of, not only MapV, but also of other related paramyxoviruses (*Hendra Virus* (HeV) and *Nipah*, (NiV), genus *Henipavirus*), MapV and *Tioman Virus* (TioPV), genus *Rubulavirus*) TioV) [4,14-18], it has been suggested that bats could harbor the origin of the PRV responsible for the BED outbreak in pigs [4]. Thus, there are many unanswered questions

Table 1: The number of specimens (per location) collected of each species of bat.

∞ Family	Species	Cave* 1	Cave* 2	No. of specimens
Mormoopidae	*Pteronotus parnellii* (Insectivorous)	9	0	9
Molossidae	*Tadarida brasiliensis* (Insectivorous)	2	0	2
Phyllostomidae	*Artibeus hirsutus* (Frugivorous)	0	15	15
	Artibeus jamaicensis (Frugivorous)	4	0	4
	Sturnira lilium (Frugivorous)		1	1
	Glossophaga soricina (Nectarivorous)	0	5	5
	Macrotus waterhousii (Insectivorous)	0	1	1
	Desmodus rotundus (hematophagous)	2	1	3
Emballonuridae	*Balantiopteryx plicata* (Insectivorous)	2	0	2
Total		19	23	42

*Bat location: N. 18º 42´ 32.4"W 99º14´14.1" 1004 MASL (cave 1); N. 18º46´14.33" W 98º 5371´ 55.95" 1403 MASL (cave 2)

concerning PRV persistence in domestic pigs and regarding possible reservoirs in nature.

Case Presentation

Objectives

- Study aimed to determine the antibodies presence of PRV in Mexican bats using a serological approach.
- Discuss the approach about that PRV could be circulating in bat populations.

Material and Methods

Bats were collected under the SEMARNAT DGVS permit FAUT-0211 following the guidelines of the Animal Care and Ethical Committee of Centro Nacional de Investigaciones Disciplinarias en Microbiología Animal, INIFAP, Mexico City. The specimens were deposited at Colección de Mamíferos del Centro de Investigación en Biodiversidad y Conservación, Universidad Autónoma del Estado de Morelos. A total of 42 bats were caught from 2 different refuges/caves. The refuges were selected in a pig fattening area where spontaneous outbreaks of BED had occurred (central zone of Mexico). The specimen was classified by experts of Centro de Investigación en Biodiversidad y Conservación. In order to determine their immune state, the bats were placed under light anesthesia, bled to death, and a necropsy was conducted to collect serum and brain samples for serological analysis and PCR respectively [19,20]. Serum samples were tested by serum-virus neutralizing analysis in cell culture (VN) and Immunoperoxidase Monolayer Assay (IPMA) to PRV. All tests were performed by standard protocols [8,11]. PCR products were sequencing and phylogenetic analysis was performed compared with viral sequences reported at the Gene bank of the *Paramyxoviridae* family [21-23].

Results

A total of 42 bats, belonging to eight different species (Table 1), were sampled from two different refuges located in the Mexican state of Morelos of the Central part of Mexico. The refuges were selected in a pig fattening area where spontaneous outbreaks of BED had occurred. Neutralizing antibodies against PRV were detected in 7 serum samples with titers ranging between 1:20 to 1:1280. IPMA confirmed the presence of antibodies against PRV in all of the VN positive bat serum, as well as showed positive result for 8 additional bat serum samples with titers ranging from 1:10 to 1:40 serum dilutions. IPMA positive samples were characterized by the presence of a few irregular structures stained red in the cytoplasm, representing inclusion-like bodies (Figure 1A-1E). Serum from the controls were positive and negative respectively (Figure 1F, 1G). Additionally, 6 out of 15 serological positive samples showed PCR positive result within the *Paramyxoviridae* family; *Avulavirus-Rubulavirus* genus (Table 2). Only a single sequence of 268 positions (access number MT636875) was correct assembly and the alignment results showed a nucleotide identity of 90.87% to PRV, 71.64% to MapV, 75.46% to *Parainfluenza virus* 5, 72.31% to *Mumps virus*, 73.58% to *Bat paramyxovirus*, 73.56% to *Tuhoko virus* 2 and 72.37% to *Menangle virus*. The phylogenetic analysis of the nucleotide sequence confirmed that the sequence is closest related to LPMV-PRV as they grouped together on a separate clade (Figure 2).

Discussion

In recent years, several novel members of the *Paramyxoviridae* family have emerged infecting both humans and/or domesticated animals. Bats have been implicated in numerous of these new emerging infectious diseases as they have been shown to harbor a great diversity of paramyxoviruses [1,15,16,24-27]. Two paramyxoviruses belonging to the subfamily *Rubulavirinae* have in retrospective analysis been

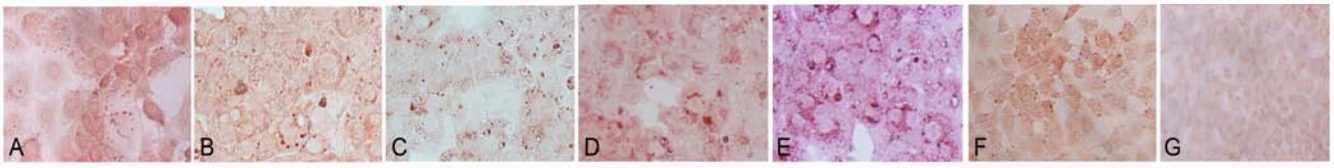

Figure 1: Detection of specific antibodies against PRV in serum bat samples by Immunoperoxidase Monolayer Assay (IPMA) in an infected MDCK cells stained by applying protein A from *Staphylococcus aureus* labeled with horseradish peroxidase and AEC substrate. (A, B) Bat 3 and 42, samples from *Desmodus rotundus,* C) Bat 16, sample from *Macrotus waterhousii*, (D, E) Bat 23 and 28, samples from *Artibeus hirsutus*, F) Positive serum control from an infected pig, 20x magnification (G) Mock infected MDCK cells, 10x magnification.

Table 2: Summary of positive bat samples by serological analysis: Serum-Virus Neutralizing in cell culture (VN), Immunoperoxidase Monolayer Assay (IPMA) and bat samples tested positive to *Paramyxoviridae* family or *Paramyxovirinae* subfamily *Avulavirus-Rubulavirus* genus by RT-PCR, semi-nested RT-PCR.

∞ Bat ID/Year collected/Cave∞	Serological Test		PCR		Species	Family	Feeding
	VN*	IPMA*	*Paramyxoviridae family*	*Paramyxovirinae subfamily Avulavirus-Rubulavirus genus*			
9/2011/Cave 1	1:320	-			*Artibeus jamaicensis*	*Phyllostomidae*	Frugivorous
10/2011/Cave 1	1:1280	1:20-	+		*Artibeus jamaicensis*	*Phyllostomidae*	Frugivorous
11/2011/Cave 1	1:20	-			*Artibeus jamaicensis*	*Phyllostomidae*	Frugivorous
13/2011/Cave 1	1:20	1:10	+		*Artibeus jamaicensis*	*Phyllostomidae*	Frugivorous
3/2013/Cave 1	1:80	1:20	-	+	*Desmodus rotundus*	*Phyllostomidae*	Hematophagus
6/2013/Cave 1	-	1:20	+	+	*Pteronotus parnellii*	*Phyllostomidae*	Insectivorous
7/2013/Cave 1	1:320	1:10	-	-	*Pteronotus parnellii*	*Phyllostomidae*	Insectivorous
11/2013/Cave 1	-	1:10	-	-	*Pteronotus parnellii*	*Phyllostomidae*	Insectivorous
13/2013/Cave 1	-	1:40	-	-	*Pteronotus parnellii*	*Phyllostomidae*	Insectivorous
16/2013/Cave 2	-	1:20	-	-	*Macrotus waterhousii*	*Mormoopidae*	Insectivorous
23/2013/Cave 2	-	1:20-	+	+	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
28/2013/Cave 2	-	1:40	+	+	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
31/2013/Cave 2	-	1:40	-	-	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
32/2013/Cave 2	-	1:20	-	-	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
33/2013/Cave 2	-	1:20	+	+	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
35/2013/Cave 2	-	1:40	-	-	*Artibeus hirsutus*	*Phyllostomidae*	Frugivorous
42/2013/Cave 2	1:320	1:20	-	-	*Desmodus rotundus*	*Phyllostomidae*	Hematophagus
TOTAL	7	15	6	5			

∞ Bat location: N. 18º 42´ 32.4"W 99º 14´ 14.1" 1004 MASL (cave 1); N. 18º 46´ 14.33" W 98º 51´ 55.95" 1403 MASL (cave 2). * = Titres are expressed as the reciprocal of the last serum dilution showed positive reaction; + = positive; - = negative

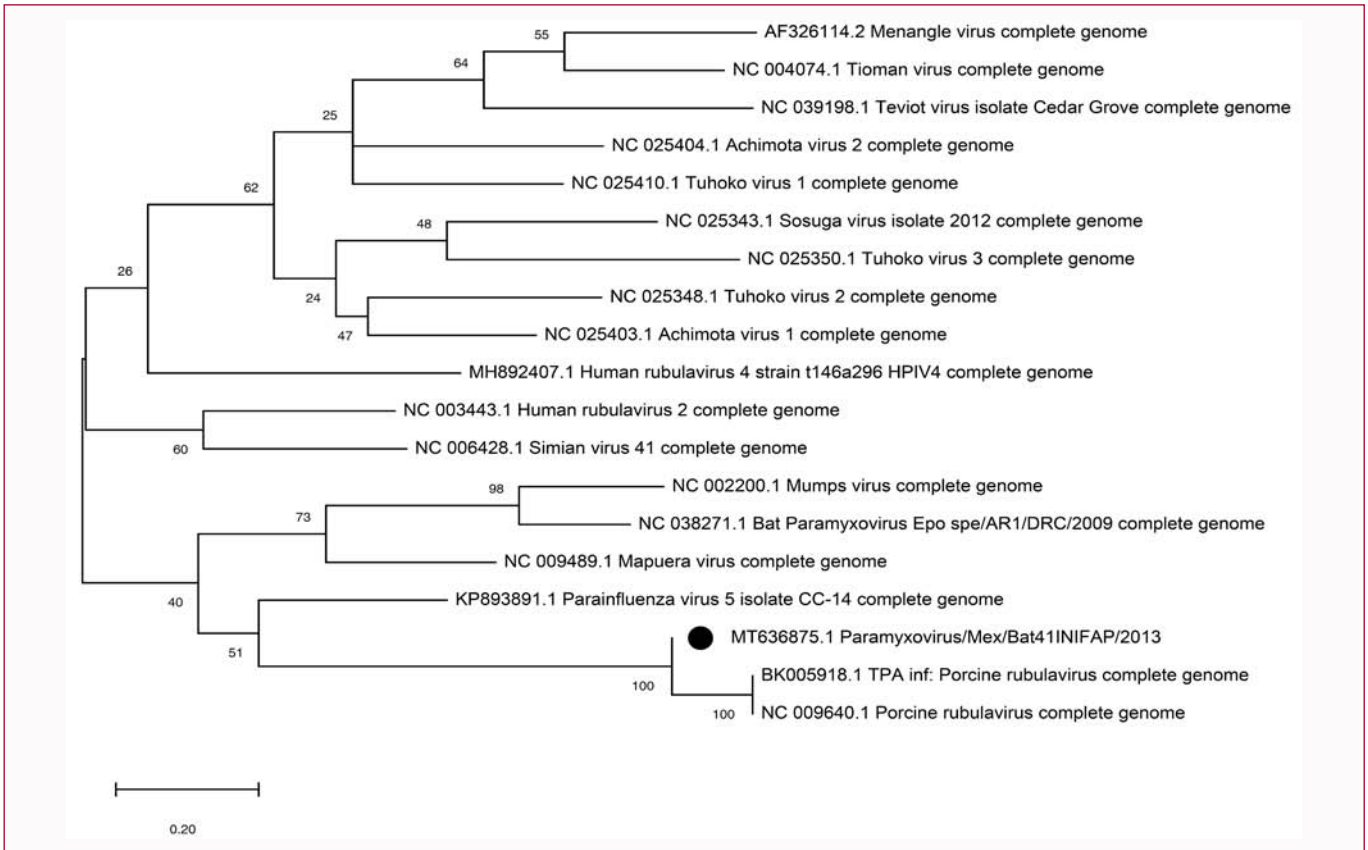

Figure 2: Phylogenetic analysis of nucleotide sequences derived from 268 positions in the final dataset of partial L gene fragment was carried out based on the best model, resulting in Tamura 3-parameter model with a discrete Gamma distribution and constructing the tree with the maximum likelihood method and 1000 bootstraps included 19 genus of the subfamily *Paramyxovirinae*.

identified on the American continent. One of them is *Mapuera virus*, that was isolated from the salivary glands of a yellow-shouldered bat (*Sturnira lilium*) captured in the tropical rainforest of Brazil in 1979 [28] and the other is a *Porcine rubulavirus* (PRV) that was isolated from pigs and that was shown to be the causative agent behind several disease outbreaks in pigs in the early 1980´s [6,7]. Molecular studies of PRV suggest that PRV has existed as a separated species for a long time in nature and that it could have been transmitted from a natural wildlife reservoir to domesticated pigs [29-31]. More recent studies of these two viruses, comparing the completed genome sequence and the genome organization, indicates that PRV is more closely related to MapV than to other members of the subfamily *Rubulavirinae* suggesting that PRV may, possibly, also been originated from bat [4]. In addition, a serological analysis in 108 non-hematophagous bats from the Central Pacific coast of Mexico, showed the presence of antibodies against PRV in insectivorous bat (*Rhogeessa parvula* major). However, due to as a single bat was seropositive; the authors suggest that a bat in the surveyed localities does not play a role in the epidemiology of PRV. Thus, in this study serological analysis showed seropositivity in 15/42 of the investigated serum samples. The percentage of seropositive bats was high compared to the study from 2004 where only 1 of 108 bats was seropositive [32]. However, the sampling in this study was done at a location in a central zone of Mexico where spontaneous outbreaks of BED occurred; which could explain the differences observed. Also all bats, in this study, were trapped in two caves containing a dense bat population of mixed species. This close coexistence may play an important role in spreading the virus in this particular bat population by allowing easier transmission between immune and naïve individuals and between different species through certain behaviors, such as mutual grooming and biting during mating [3]. Thus the presence of anti-PRV antibodies in the bat specimens, suggests that PRV is indeed circulating in bat populations. However, we cannot rule out that this is a related virus with cross-reactivity to PRV. In addition, some seropositive bat samples were PCR negative, it could be speculated that these individuals could carry the virus in other organs not tested in this study. The phylogenetic analysis of the unique aligned sequence, grouped together with PRV-LPMV, suggesting that they could belong to the same species of the *porcine orthorubulavirus*.

Conclusion

These findings provide serological evidence that bats could act as an intermediate or natural host of PRV. However, future studies are required to investigate the infection in bats, to determine the exact role of bats in PRV epidemiology, the tropism and the behavior of the virus in nature. Also further genetic analysis could be valuable to understand the evolution of the virus.

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