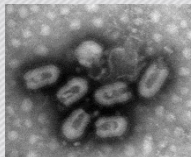




LA NOSTRA  
ESPERIENZA,  
LA VOSTRA  
**SICUREZZA.**



Davide Lelli

## I CHIROTTERI COME OSPITI RESERVOIR DI VIRUS ZONOTICI EMERGENTI: FLAVIVIRUS E NON SOLO



# Summary



- Bats ecology
- Bats and viruses
  - Arthropod-borne viruses: Flaviviruses
- Project code GR-2011-023505919
  - Methods
    - Sampling
    - Virological tests
    - Molecular characterization
    - Phylogenetic analysis
  - Results
  - Conclusions





# Introduction - Bats



## Bats

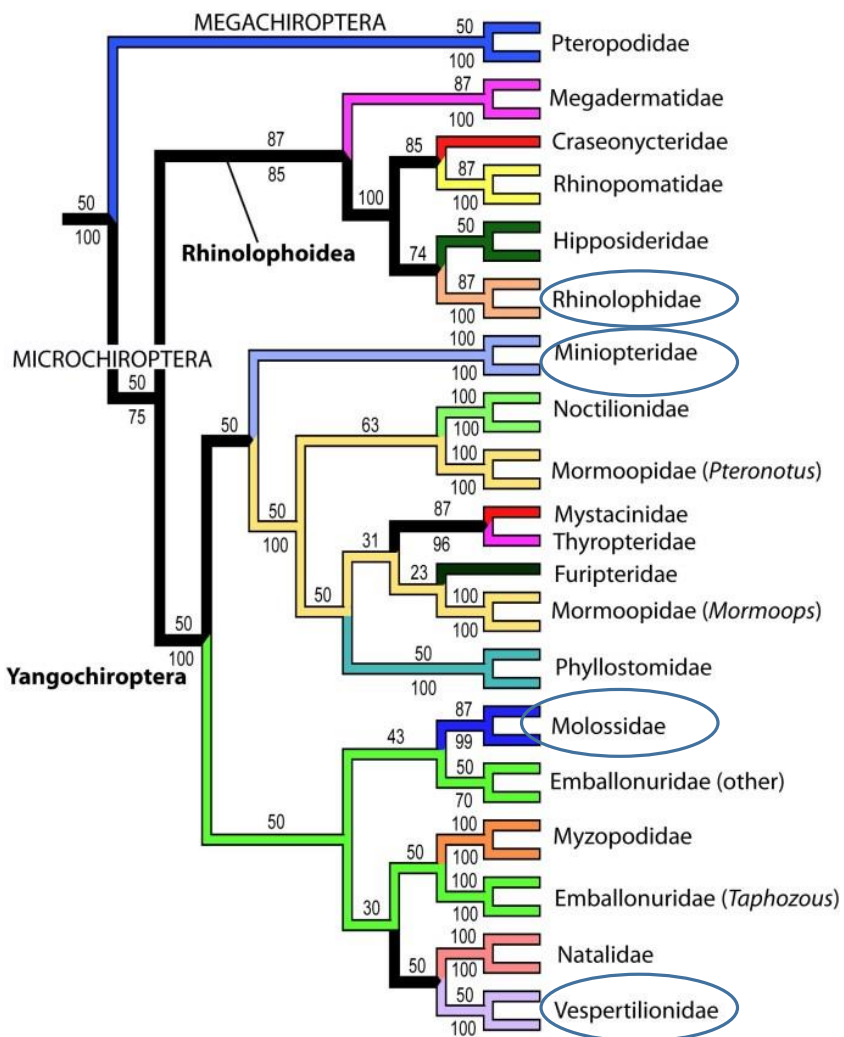
- Order of Chiroptera,  
one of the largest groups of mammals (>1200 species)
- Two suborder:  
*Microchiroptera* and *Megachiroptera*  
Yangochiroptera - Yinpterochiroptera
- Large diversity of diets
- In Europe 53 species (*Microchiroptera*)







# Introduction - Bats



34 species  
belonging to 4 families

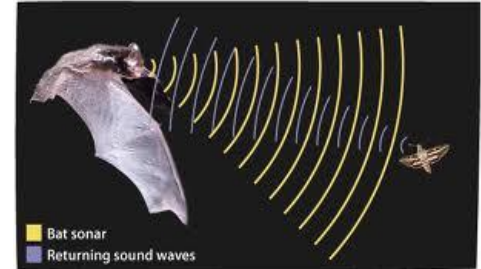
Agnarsson I et al., 2011. *PLoS Curr* 4;3:RRN1212.



# Introduction - Bats



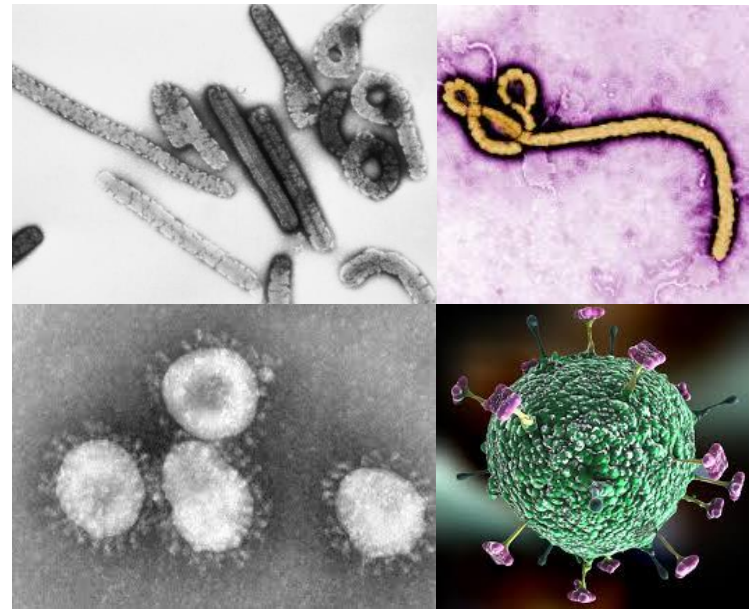
- ✓ EUROBATS
  - Pest-controlling actions
  - Biodiversity
  - Several species are vulnerable or threatened
- ✓ Low reproductive ratio
- ✓ High longevity
- ✓ Bats are the only mammals that can fly
- ✓ They are migratory
- ✓ Torpor and hibernation during winter
- ✓ They are all echolocators
- ✓ Bats are the most social mammals
- ✓ Bats are nocturnal
- ✓ Unique immunology and spatial population structure





## *Why bats?*

- ✓ Bats are increasingly recognized as reservoir/hosts for a large number of zoonotic emerging viruses
- ✓ Little is known on similar human-pathogenetic viruses that may be present in European bats
- ✓ Bats are poorly studied in Italy for virologic aspects

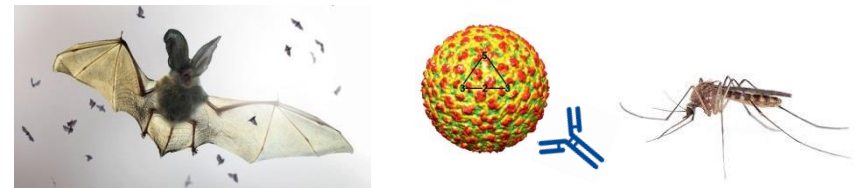
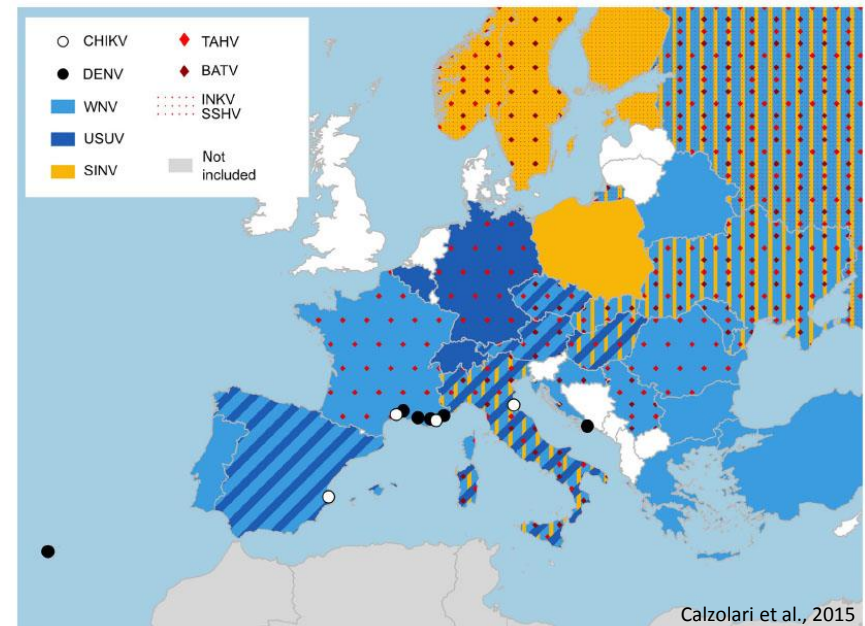






## Why Arboviruses (flavivirus)?

- ✓ The **diet and habits of insectivorous bats** may contribute to infection by mosquito-borne viruses
- ✓ Arboviruses may infect bats **via arthropods**, but it is not clear whether bats are reservoir/hosts for these viruses
- ✓ **Oral route of infection** (bats might become infected in nature by feeding on infected mosquitoes)
- ✓ Survey of insectivorous bats could allow a better understanding of the ecology of these viruses.





# Mosquito-borne flaviviruses



## JEV

*La Motte LC, 1958:* suggested a **mosquito-bat-mosquito cycle** as a possible **overwintering** mechanism for JEV.

- **Oral route of infection** (bats might become infected in nature by feeding on infected mosquitoes)
- Infected mosquitoes can transmit the virus (JEV) from and to bats under the lab conditions.

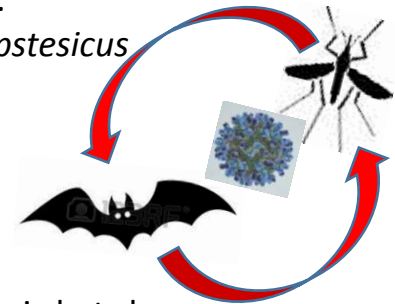
*Sulkin SE et al., 1963, 1964, 1966.* Subcutaneously inoculation of JEV in *Tadarida brasiliensis*, *Epstesicus Fuscus* and *Myotis lucifugus*.

- **Viremia** lasted for 15 to 30 days;
- Neutralizing Ab appeared as the viremia declined;
- Virus isolation (during the period of viremia) from brains, kidneys and **brown fat**;
- **transplacental passage** of JEV to the offspring in *Tadarida brasiliensis*.

*Banerjee K et al., 1984. Indian J Med Res 79:8.* Demonstrated the Transmission of JEV from viremic bats by *Culex bitaeniorhynchus* to bats and chicks

*Miura T. et al., 1970.* Serological evidence of JEV natural infection in different bat species from several countries

*Wang JL et al., 2009. Emerg Infect Dis. 15:939-42.*



## SLEV

*Herbold JR et al., 1983:* Inoculation and hibernation of big brown bats.

- SLEV was maintained in bats through **hibernation** (70 days)
- **Viremia** within 4 days of arousal from hibernation (105 days p.i)
- 9% prevalence rate of neutralizing antibody to SLE in nature.
- Bat may be involved in the maintenance of SLE virus in enzootic foci and could have a role in dissemination of SLE virus to epizootic foci

*Sulkin SE et al., 1963, 1964:* **transplacental passage** of SLEV in *Myotis lucifugus*.





# WNV e USUV in bats

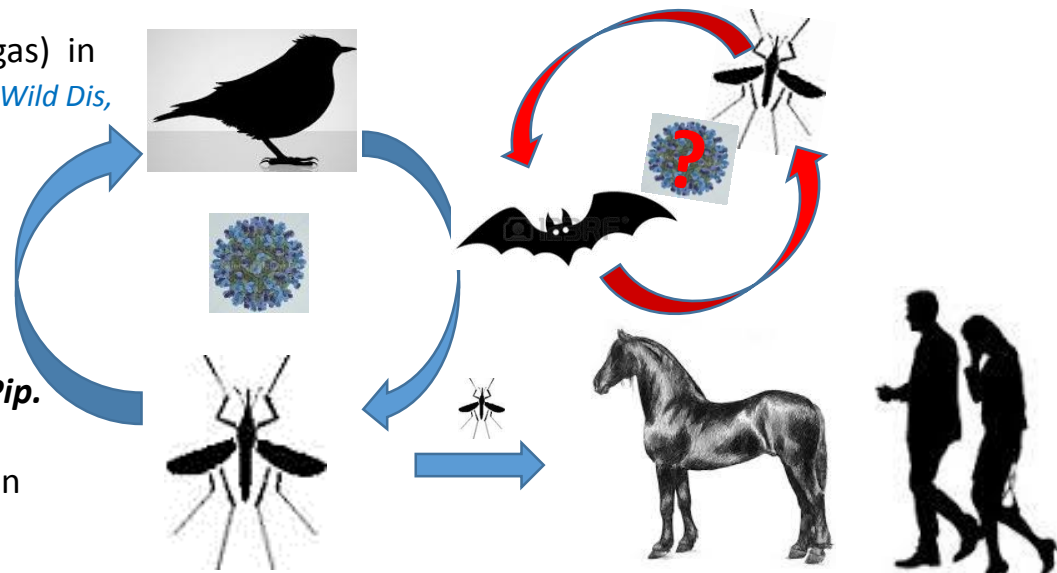


## WNV

- Isolation OF wnv from the **spleen** of one **frugivorous bat**, *Rousettus leschenault* (Paul SD et al., 1970. *Indian J Med Res*, 58(9):1169-71).
- Neutralizing and WNV Ab have been reported from different bat species (*Eptesicus fuscus*, *Myotis lucifugas*) in USA (Marfin AA et al., 2001. *EID* 7:730-35; Pilpski JD. 2004, *J Wild Dis*, 40:335-40)

## USUV

- PCR positivity in brain samples of two dead bats (*Pip. Pipistrellus*) in Germany in 2013.
- bats probably contribute to the epizootic rather than act as a silent reservoir for the virus.
- USUV might have a higher **tropism for the nervous system** in bats, as opposed to the pantropism observed in birds (Cadar D, et al., 2014. *EID*, 20:1771-3)



**The susceptibility of bats to infection and the level of viral replication varied with different viral strains and the species of bats tested.**



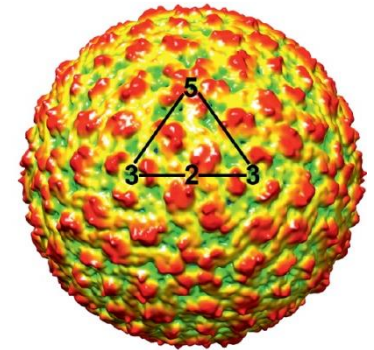
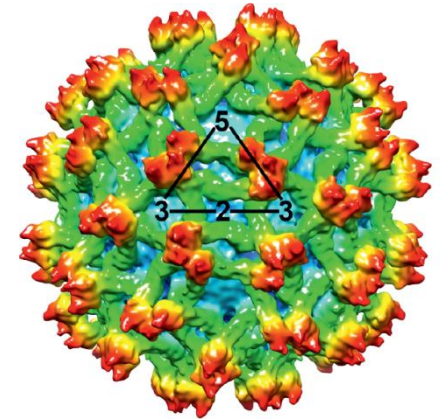
# Flavivirus



Family **Flaviviridae** ssRNA, 40-60 nm, envelope

## *Genus Flavivirus*

- ✓ Mosquito-borne flaviviruses
- ✓ Tick-borne flaviviruses
- ✓ Mosquitoes-only Flaviviruses (Mofs)
- ✓ No known vector flavivirus (NKV)
- ✓ Tentative species in the genus





# No known vector flavivirus (NKV)



## Bat-associated no known vector flavivirus (NKV)

Table 1. Geographic distribution, natural host range and clinical manifestations of bat-associated no known vector (NKV) flaviviruses.

Virus	Human Disease	Year of First Isolation	Geographic Distribution	Natural Host Range <sup>a</sup>	References
Batu Cave virus (BCV) <sup>b</sup>	No	1971	Malaysia	<i>Cynopterus brachyotis</i> (lesser short-nose fruit bat), <i>Eonycteris spelaea</i> (dawn bat)	[18,19]
Bukalasa bat virus (BBV)	No	1963	Senegal, Uganda	<i>Chaerephon pumila</i> (little free-tailed bat), <i>Tadarida (Mops) condylurus</i> (Angolan free-tailed bat)	[20–23]
Carey Island virus (CIV)	No	1970	Malaysia	<i>Cynopterus brachyotis</i> (lesser short-nosed fruit bat), <i>Macroglossus lagochilus</i> (lesser long-tongued fruit bat)	[24]
Dakar bat virus (DBV)	Yes (fever)	1962	Central African Republic, Madagascar, Senegal, Nigeria, Uganda	<i>Chaerephon pumilus</i> (little free-tailed bat), <i>Scotophilus nigrita</i> (giant house bat), <i>Tadarida (Mops) condylurus</i> (Angolan free-tailed bat), <i>Taphozous perforatus</i> (Egyptian tomb bat), <i>Homo sapiens</i> (human)	[20,21,23–26]
Entebbe bat virus (ENTV) <sup>c</sup>	No	1957	Uganda	<i>Chaerephon (Tadarida) pumilus</i> (little free-tailed bat) <sup>d</sup>	[21,27,28]
Montana myotis leukoencephalitis virus (MMLV)	No	1958	United States	<i>Myotis lucifugus</i> (little brown bat)	[29]
Phnom Penh bat virus (PPBV)	No	1969	Cambodia, Malaysia	<i>Cynopterus brachyotis</i> (Lesser short-nosed fruit bat), <i>Eonycteris spelaea</i> (dawn bat)	[24,30]
Rio Bravo virus (RBV) <sup>e</sup>	Yes (fever)	1954	United States, Mexico, Trinidad	<i>Eptesicus fuscus</i> (big brown bat), <sup>f</sup> <i>Molossus rufus</i> (black mastiff bat), <i>Tadarida brasiliensis mexicana</i> (Mexican free-tailed bat)	[24,31–35]
Sokoluk virus (SOKV) <sup>g</sup>	No	1970	Kyrgyzstan, Russia	<i>Pipistrellus</i> spp. bats, <i>Argasidae</i> spp. ticks	[36,37]
Tamana bat virus (TABV) <sup>h</sup>	No	1973	Trinidad	<i>Pteronotus parnellii</i> (Parnell's mustached bat)	[32]
Yokose virus (YOKV)	No	1971	Japan	<i>Miniopterus fuliginosus</i> (eastern bent-wing bat)	[38]

<sup>a</sup> Restricted to species that have yielded isolates (serological data not considered); <sup>b</sup> Subtype of Phnom Penh bat virus; <sup>c</sup> Formerly known as Entebbe bat salivary gland virus; <sup>d</sup> Also known as *Chaerephon (Tadarida) limbata*; <sup>e</sup> Formerly known as bat salivary gland virus; <sup>f</sup> Also known as *Molossus ater*; <sup>g</sup> subtype of Entebbe bat virus; <sup>h</sup> Not classified as a NKV flavivirus by the International Committee on Taxonomy of Viruses (ICTV); it has been tentatively assigned to the genus *Flavivirus* and has no known arthropod vector.


Blitvich BJ, Firth AE. 2017. A Review of Flaviviruses that Have No Known Arthropod Vector. *Viruses*, 9:154.





# PGR 2011001



 <p><i>Ministero della Salute</i> Direzione Generale della Ricerca Sanitaria e Biomedica e della Vigilanza sugli Enti BANDO 2011-2012 PROGETTI DI RICERCA PROGETTO COMPLETO</p>	<p>Project Title: An epizootiological survey of bats as reservoirs of emerging zoonotic viruses in Italy: implications for public health and biological conservation</p>
<p>Project Code: GR-2011-02350591</p>	<p>Principal Investigator: Davide Lelli</p>
<p>Research Type: Biomedical/Biomedica</p>	<p>Applicant Institution: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia</p>
<p>Project Type: Young Researcher (under 40 years)/Giovani Ricercatori</p>	



Istituto Zooprofilattico Sperimentale della  
Lombardia e dell'Emilia-Romagna  
"B. Ubertini"

## U01

Responsabile scientifico: **Davide Lelli**

*Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*



## U02

Responsabile scientifico: **Paola Debenedictis**

*Istituto Zooprofilattico Sperimentale delle Venezie*

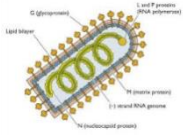


## U03

Responsabile scientifico: **Nicola Decaro**

*Università degli Studi di Bari, Facoltà di Medicina Veterinaria*

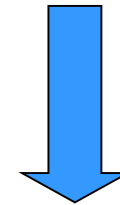




# METHODS



Bats are protected by Agreement on the Conservation of Populations of European Bats (EUROBATS)



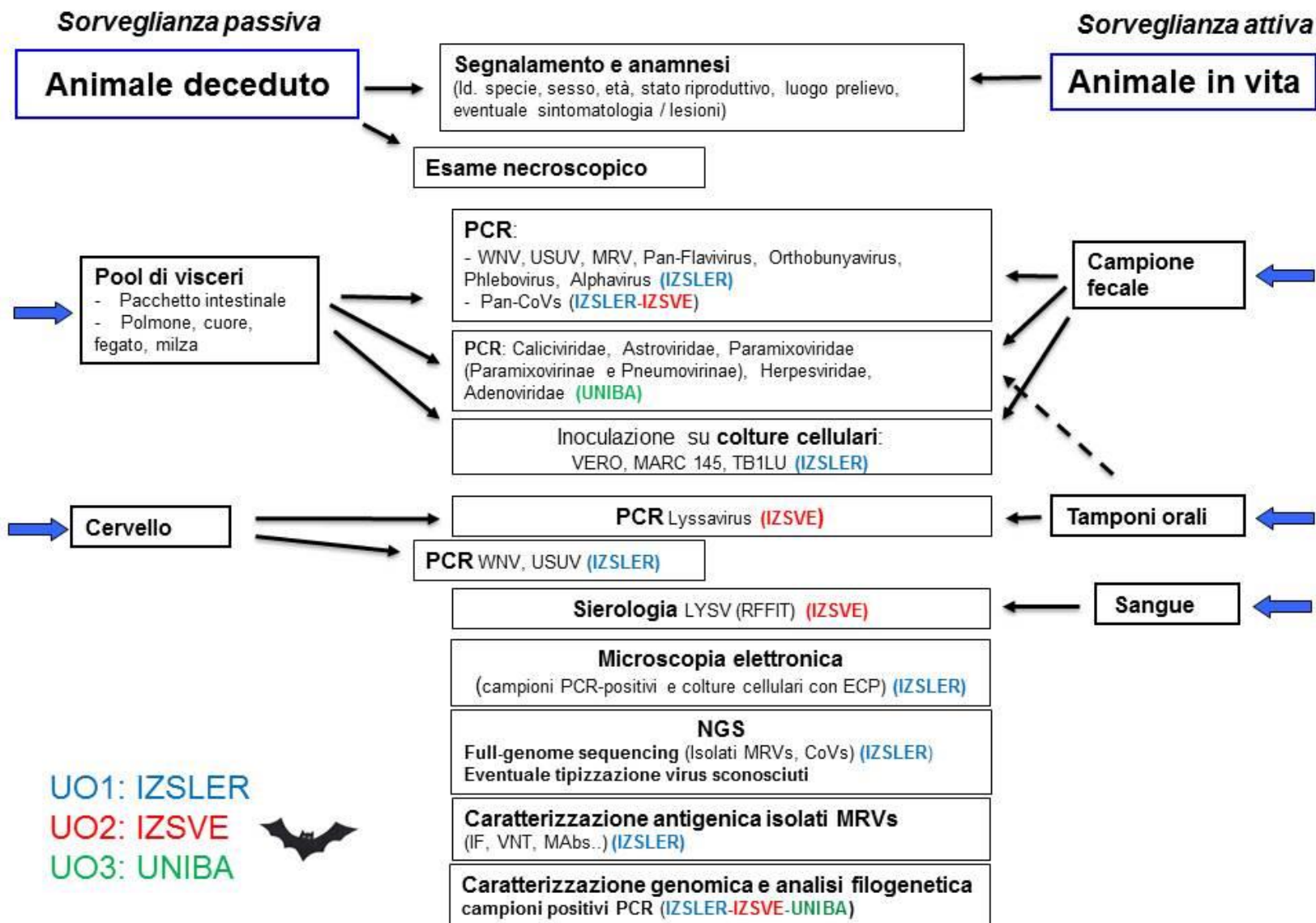
Non-killing techniques and invasive sampling were used



# Methods – Diagnostic protocol



## PGR 2011-02350591- Protocollo diagnostico







# Methods - Sampling



Faecal samples from living animals



Wild Animal Recovery Centres, Bat Rehabilitation Facilities



Collection of recently dead individuals



Known reproductive and swarming sites

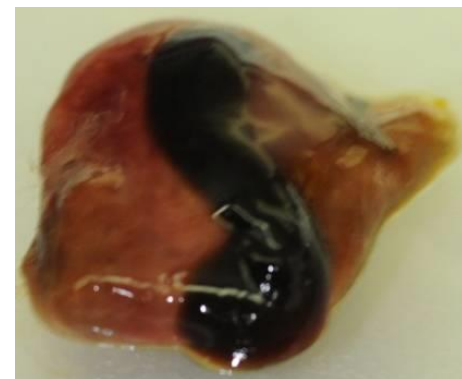


**Samples were immediately stored at -20° C and conferred to the Virology Lab for diagnostic investigations**



# Methods - Post-mortem examination

The **bat species** were identified based on their morphologic characteristics



- ✓ Brain
- ✓ Pool of viscera: lung, heart, spleen and liver
- ✓ Intestine





# Methods - Laboratory analysis



## Faecal and organ samples

Homogenized in MEM (1g/10ml) containing antibiotics and clarified by centrifugation



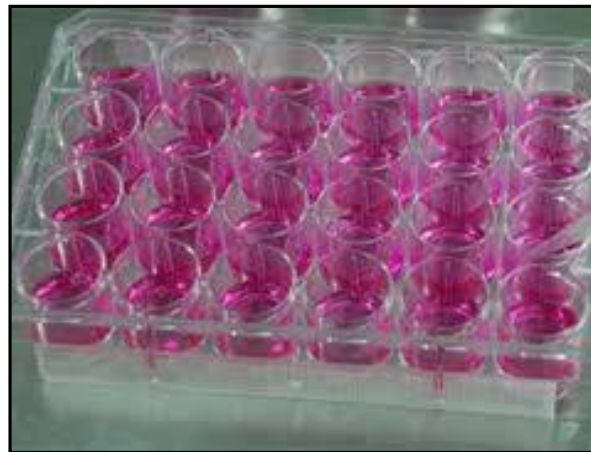
Viral RNA extraction

## Negative staining Electron microscopy

- ✓ Airfuge method
- ✓ Direct examination of samples or supernatants of cell culture with CPE
- ✓ Morphological identification of viral particles



## Cells culture



- ✓ MARC 145  
(Foetal monkey kidney cells)
- ✓ VERO  
(African green monkey kidney cells)
- ✓ TB1-Lu  
(Bat lung cell Line, Tadarida brasiliensis)
- ✓ LLC-MK2  
(Rhesus Monkey Kidney Epithelial Cells)





# Methods - Laboratory analysis

## PCR protocols

Organs and fecal samples were examined through a **broadly reactive PCR-based protocol** for the presence of viral agents with zoonotic potential:

- ✓ Coronaviruses
- ✓ Lyssaviruses
- ✓ Orthoreoviruses
- ✓ **Flaviviruses**
  - WNV
  - USUV
- ✓ **Alphaviruses**
- ✓ **Orthobunyaviruses**
- ✓ Astroviruses
- ✓ Herpesviruses
- ✓ Adenoviruses
- ✓ Paramyxoviruses
- ✓ Pneumovirus
- ✓ Caliciviruses

Arboviruses





# Methods - Laboratory analysis



## Sequencing and Molecular analysis

- ✓ **Sequencing** of PCR products
- ✓ **Blast analysis and phylogenetic trees** for each genome segment were performed
- ✓ **Ion Torrent and Illumina NGS** platform were used to obtain the whole genome sequences of selected viruses
- ✓ **The recombination detection program, RDP4**, was used to investigate the presence of genetic recombination







# Methods – Sampling



## Active surveillance



Live individual sampling from mixed maternity colonies of *Myotis myotis* and *Myotis blythii*

- Blood
- Oral swabs
- Feces
- Wing biopsy

**Samples were immediately stored at -20°/-80° C**





# Methods – Laboratory analyses

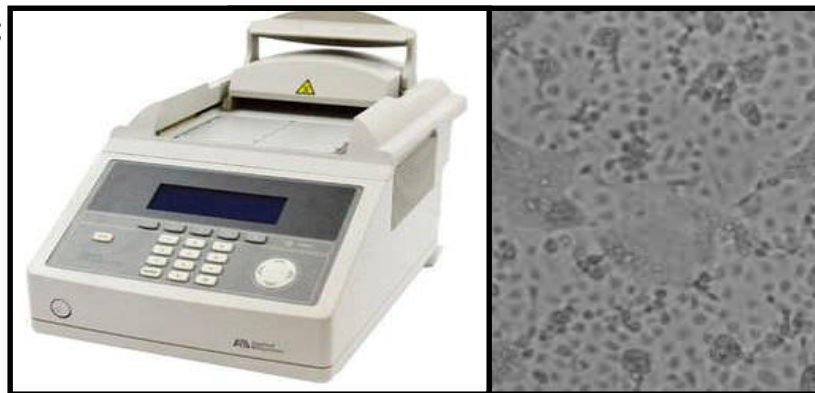
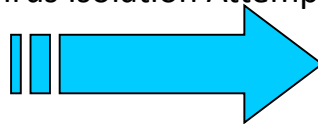


## Active surveillance

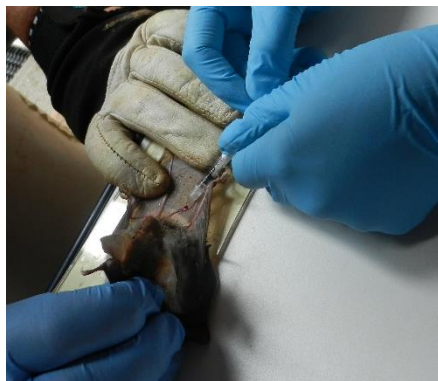
Feces/  
saliva



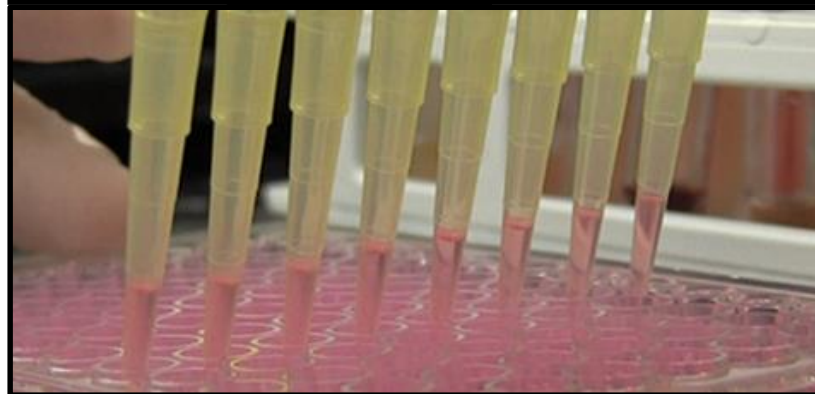
Pan CoV hemi nested RT-PCR;  
pan LysV RT-PCR;  
Virus Isolation Attempt



Blood



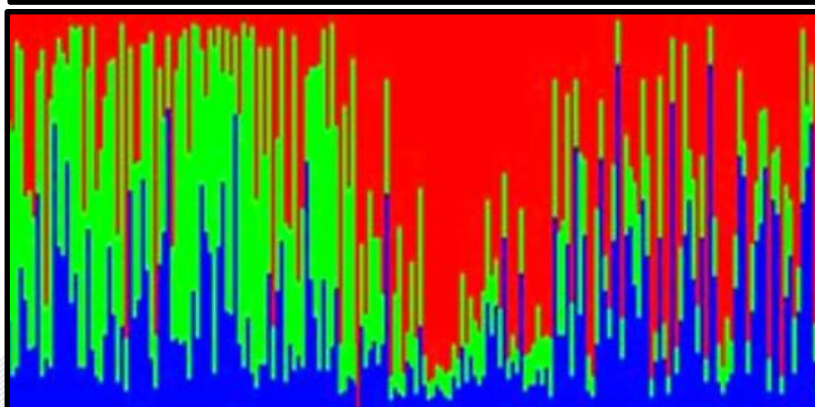
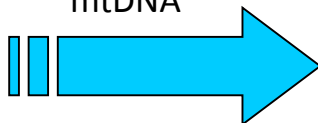
Rapid Fluorescent  
Foci Inhibition Test



Wing  
Biopsy



nuDNA and  
mtDNA





# Results





# Results - Sampling



Passive surveillance

**633 faecal and tissue samples**

From 2010 to 2017 Update 24.05.2017

Host species	Carcasses	Faeces
	N°	N°
<i>Pipistrellus kuhlii</i>	242	117
<i>Pipistrellus spp.</i>	118	11
<i>Tadarida teniotis</i>	2	1
<i>Nyctalus noctula</i>	7	1
<i>Hypsugo savii</i>	70	20
<i>Plecotus auritus</i>	12	1
<i>Vespertilio murinus</i>	4	/
<i>Pipistrellus pipistrellus</i>	1	2
<i>Rhinolophus hipposideros</i>	/	18
<i>Miniopterus schreibersii</i>	/	6
Total samples	<b>456</b>	<b>177</b>





# Results - Post-mortem findings



Mild to severe traumatic injuries:

- ✓ Lacerations of wing membrane
- ✓ Fractures

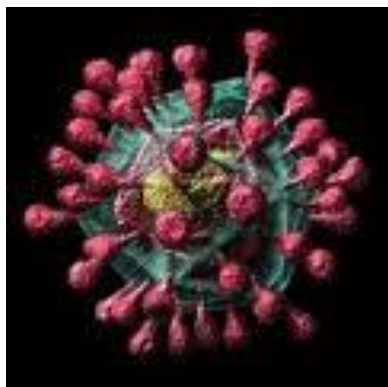
Pathological lesions were macroscopically hardly visible:

- ✓ Mild hemorrhagic or catarrhal enteritis
- ✓ Pneumonia
- ✓ Traumatic lesions



## Pan-coronavirus RT-PCR – RdRp gene

Lelli D, Papetti A *et al.*, 2013. Detection of coronaviruses in bats of various species in Italy. *Viruses*, 5:2679-2689



### 23 samples CoV POSITIVE (6,2\* %)



*Pipistrellus Khulii*



*Hypsugo savii*



*Nyctalus noctula*



*Rhinolophus hipposideros*

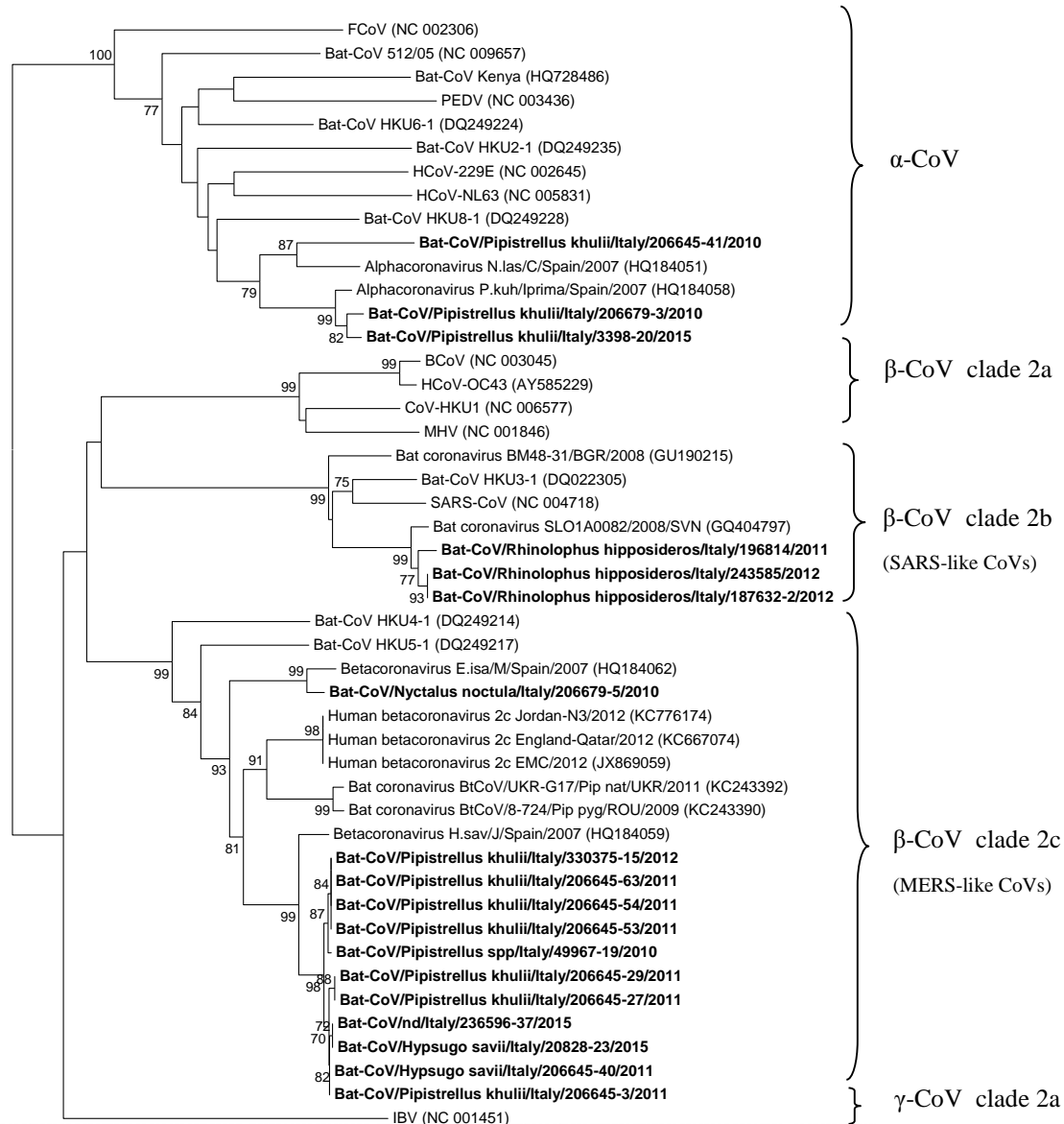
\* Prevalenza calcolata su 369 campioni testati al 07/04/2016



# Phylogenetic tree - CoVs



Phylogenetic tree of the partial RNA-dependent RNA polymerase (RdRp) gene (385 bp) of coronavirus (CoV) strains found in Italian bats. Viruses detected in this study are in bold



0.05







## Lyssaviruses



All sample resulted negative



# Results - Cells culture



Passive surveillance

## 32 MRVs were isolated by cell culture

*Pipistrellus Kuhlii*



20 MRVs

*Vespertilio murinus*



1 MRV

*Rhinolophus hipposideros*



1 MRV

*Tadarida teniotis*



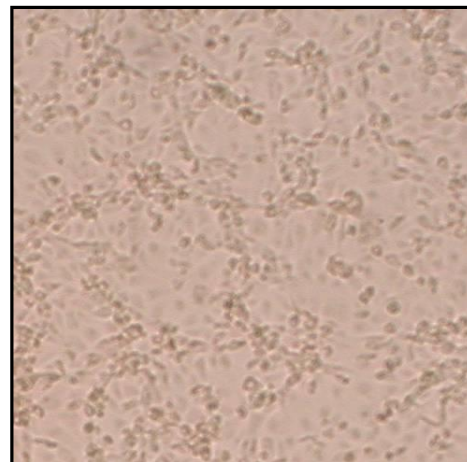
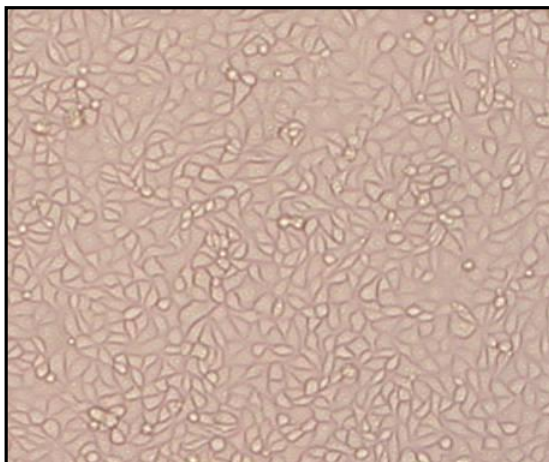
2 MRVs

Cytophatic effect on infected MARC 145/ VERO

*Hypsugo savii*



3 MRVs



*Nyctalus noctula*



1 MRV

*Pipistrellus spp*

4 MRV



**BatMRV**





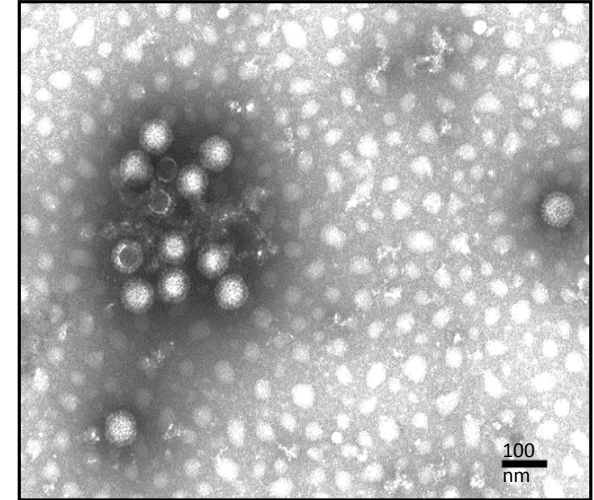


# Results - Virus identification



## Negative staining Electron Microscopy

- ✓ Presence of many icosahedral viral particles without envelope, morphologically related to **(Ortho)reovirus**
- ✓ Identical viral particles were observed by direct examination of faecal homogenate



## PCR and NGS

- ✓ The definitive assignment of the isolates to MRV was confirmed by RT-PCR assays [Leary *et al.* 2002] and NGS
- ✓ **24 confirmed as MRVs Type 3 (PCR-S1 gene)**
- ✓ **3 MRVs confirmed as MRVs Type 2**
- ✓ **1 MRV confirmed as MRVs Type 1**



# Introduction – Background literature



SPECIAL ISSUE – BATS **Zoonoses** AND PUBLIC HEALTH

## Identification of Mammalian Orthoreovirus Type 3 in Italian Bats

D. Lelli, A. Moreno, A. Lavazza, M. Bresaola, E. Canelli, M. B. Boniotti and P. Cordioli

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER, Brescia, Italy



**Journal of Clinical Microbiology**

(2013), 51(11):3818-25.

## High Similarity of Novel Orthoreovirus Detected in a Child Hospitalized with Acute Gastroenteritis to Mammalian Orthoreoviruses Found in Bats in Europe

Andrej Steyer,<sup>a</sup> Ion Gutiérrez-Aguirre,<sup>b,\*</sup> Marko Kolenc,<sup>a</sup> Simon Koren,<sup>c</sup> Denis Kutnjak,<sup>b</sup> Marko Pokorn,<sup>d</sup> Mateja Poljšak-Prijatelj,<sup>a</sup> Nejc Rakčič,<sup>b</sup> Maja Ravnikar,<sup>b,\*</sup> Martin Sagadin,<sup>a</sup> Adela Fratrik Steyer,<sup>a</sup> Nataša Toplak<sup>c</sup>

OPEN ACCESS Freely available online

PLOS ONE

## Isolation and Characterization of Three Mammalian Orthoreoviruses from European Bats

Claudia Kohl<sup>1\*</sup>, René Lesnik<sup>1</sup>, Annika Brinkmann<sup>1</sup>, Arnt Ebinger<sup>1</sup>, Aleksandar Radonić<sup>1</sup>, Andreas Nitsche<sup>1</sup>, Kristin Mühldorfer<sup>2</sup>, Gudrun Wibbelt<sup>2</sup>, Andreas Kurth<sup>1</sup>

<sup>1</sup> Robert Koch Institute, Centre for Biological Security 1, Berlin, Germany, <sup>2</sup> Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany



## A Novel Pathogenic Mammalian Orthoreovirus from Diarrheic Pigs and Swine Blood Meal in the United States

Athmaram Thimmasandra Narayanappa,<sup>a</sup> Harini Sooryanarain,<sup>a</sup> Jagadeeswaran Deventhiran,<sup>a</sup> Dianjun Cao,<sup>a</sup> Backtyalakshmi Ammayappan Venkatachalam,<sup>a</sup> Devatah Kambiranda,<sup>b</sup> Tanya LeRoith,<sup>a</sup> Connie Lynn Heffron,<sup>a</sup> Nicole Lindstrom,<sup>a</sup> Karen Hall,<sup>a</sup> Peter Jobst,<sup>a</sup> Cary Sexton,<sup>c</sup> Xiang-Jin Meng,<sup>a</sup> Subbiah Elankumaran<sup>a</sup>

Lelli et al. *Virology Journal* (2016) 13:139

SHORT REPORT

Open Access



## First identification of mammalian orthoreovirus type 3 in diarrheic pigs in Europe

Davide Lelli<sup>1\*</sup>, Maria Serena Beato<sup>2†</sup>, Lara Cavicchio<sup>2</sup>, Antonio Lavazza<sup>1</sup>, Chiara Chiapponi<sup>1</sup>, Stefania Leopardi<sup>2</sup>, Laura Baioni<sup>1</sup>, Paola De Benedictis<sup>2</sup> and Ana Moreno<sup>1</sup>



*Viruses* 2015, 7, 5844–5854; doi:10.3390/v7112908

## Detection and Characterization of a Novel Reassortant Mammalian Orthoreovirus in Bats in Europe

Davide Lelli<sup>1,\*</sup>, Ana Moreno<sup>1</sup>, Andrej Steyer<sup>2</sup>, Tina Naglič<sup>2</sup>, Chiara Chiapponi<sup>1</sup>, Alice Prosperi<sup>1</sup>, Francesca Faccin<sup>1</sup>, Enrica Sozzi<sup>1</sup> and Antonio Lavazza<sup>1</sup>

PLOS ONE 2015 Mar 17;10(3):e0118598

## Isolation and Identification of a Natural Reassortant Mammalian Orthoreovirus from Least Horseshoe Bat in China

Lihua Wang<sup>1,2</sup>, Shihong Fu<sup>1,2</sup>, Lei Cao<sup>1,2</sup>, Wenwen Lei<sup>1,2</sup>, Yuxi Cao<sup>1,2</sup>, Jingdong Song<sup>1,2</sup>, Qing Tang<sup>1,2</sup>, Hailin Zhang<sup>3</sup>, Yun Feng<sup>3</sup>, Weihong Yang<sup>3</sup>, Guodong Liang<sup>1,2,\*</sup>

JGV

Journal of General Virology

(2015), 96, 3525-3531

## Isolation and identification of bat viruses closely related to human, porcine and mink orthoreoviruses

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# Results – representatives MRV isolates



## BatMRV1-IT2011 Type 1

44°17'46,36"N 11°7'3,79"E Vergato province of Bologna, northern Italy



lesser horseshoe bat (*Rhinolophus hipposideros*)



## BatMRV2-5515/3 Type 2

## BatMRV3-5515/2 Type 3



Palazzo Santa Margherita, Biblioteca Delfini, Modena



*Pipistrellus kuhlii*







# Results - Molecular analysis



**BLAST analysis. Highest nucleotide identities for each gene segment of the BatMRV1**

BatMRV1-IT2011	% similarity	MRV strain	Serotype	Host	Disease	Country	GenBank Accession No.
L1	93	MRV-HLJ/2007	3	Pig	Fever, respiratory illness	China	HQ642769.1
	90	Porcine reovirus SHR-A	1	Pig	NA	China	JX415466.1
L2	91	Austria/729	2	Pig	Encephalitis	Austria	JN799427.1
	90	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412756.1
	90	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154725.1
L3	95	MRVTou05	2	Human	Encephalitis	France	GU196308.1
	94	MRV-HLJ/2007	3	Pig	Fever, respiratory illness	China	HQ642769.1
M1	98	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412758.1
	98	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154727.1
M2	92	MRV-HLJ/2007	3	Pig	Fever, respiratory illness	China	HQ642773.1
	92	4 Ndelle virus	Putative 4	Mouse	NA	Cameroon	AF368034.1
M3	93	MRVTou05	2	Human	Encephalitis	France	GU196314.1
	92	Austria/729	2	Pig	Encephalitis	Austria	JN799425.1
S1	90	T1/bovine/Maryland/Clone23/59	1	Bovine	NA	USA	AY862134.1
	88	C/bovine/Indiana/MRV00304/2014	1	Bovine	Diarrhea	USA	KJ676385.1
S2	95	China/MPC/04	3	Civet	NA	China	GQ468273.1
	94	T3/bat/Germany/342/08	3	Bat	Hemorrhagic enteritis	Germany	JQ412762.1
	94	SI-MRV01	3	Human	Acute gastroenteritis	Slovenia	KF154731.1
	94	MRV-HLJ/2007	3	Pig	Fever, respiratory illness	China	HQ642776.1
S3	91	SC-A	3	Pig	Diarrhea	China	DQ411553.1
	91	Feline/California/Cornell/1968	3	Cat	NA	USA	U35362
S4	95	MRVTou05	2	Human	Encephalitis	France	GU196313.1
	95	MRV-HLJ/2007	2	Pig	Fever, respiratory illness	China	HQ642778.1

Note: L, large segments; M, medium segments; S, small segments; NA, not available



# Results - Molecular analysis



**BLAST analysis. Highest nucleotide identities for each gene segment of the BatMRV3**

BatMRV1-IT2011	% similarity	MRV strain	Serotype	Host	Disease	Country	GenBank Accession No.						
<b>L1</b>	99	T3/bat/Germany/342/08	<b>3</b>	Bat	Haemorrhagic enteritis	Germany	JQ412755.1						
							KF154724.1						
							<b>L2</b>	JQ412756.1					
								KF154725.1					
							<b>L3</b>	JQ412757.1					
								KF154726.1					
							<b>M1</b>	SI-MRV01	<b>3</b>	Human	Acute gastroenteritis	Slovenia	JQ412758.1
							<b>M2</b>						KF154727.1
							<b>M3</b>						JQ412759.1
													KF154728.1
<b>S1</b>	JQ412760.1												
	KF154729.1												
<b>S4</b>	JQ412761.1												
	KF154730.1												
<b>S3</b>	JQ412764.1												
	KF154733.1												
<b>S2</b>	98	JQ412763.1											
	99	T3/bat/Germany/342/08	Bat	Hemorrhagic enteritis	Germany	JQ412762.1							
	97	MRV-HLJ/2007	Pig	Fever, respiratory illness	China	HQ642776.1							
	94	SI-MRV01	Human	Acute gastroenteritis	Slovenia	KF154731.1							

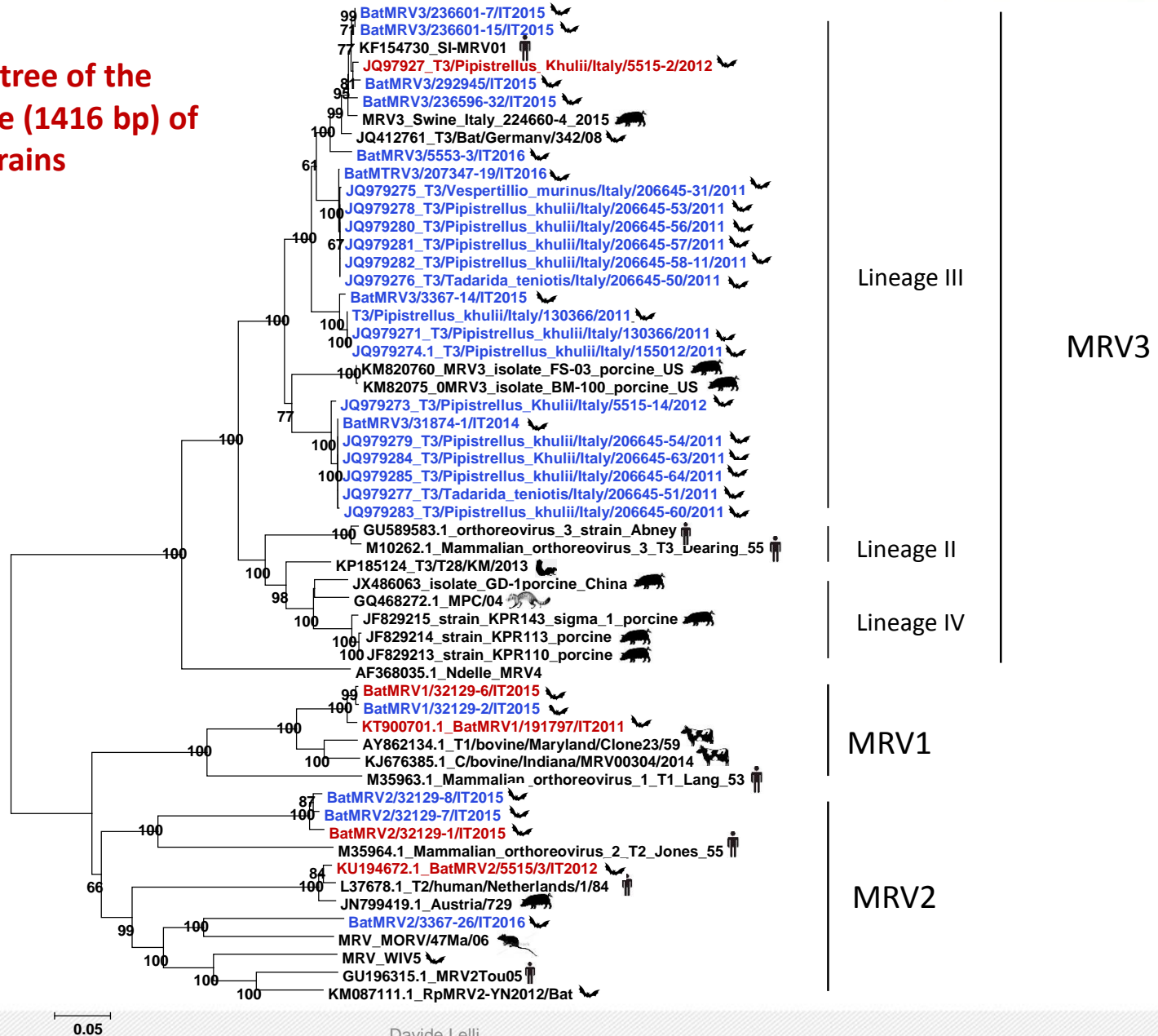
Note: L, large segments; M, medium segments; S, small segments; NA, not available



# Results - Molecular analysis



Phylogenetic tree of the complete S1 gene (1416 bp) of MRV strains







# Laboratory analysis



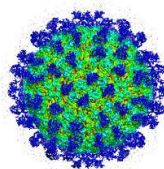
Flaviviruses

Alphaviruses

Orthobunyavirus

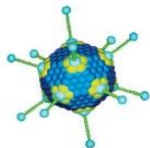


Arthropod-borne viruses



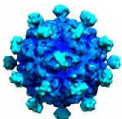
**All sample resulted negative**

Adenovirus



3367-16/2015: *Hypsugo savii* (F J, Visceri, Castel nuovo Rangone)

Astrovirus



3398-25/2015 *Pipistrellus kuhlii* (F J, Nonantola, Mink/like)  
3398-29/2015 *Pipistrellus kuhlii* (F J, Modena, Bat/like)



# Results - Sampling

## Active surveillance



## Sampling campaigns 2015 and 2016

Colony	Host species	Blood	Saliva	Faeces	Wing biopsy
		N°	N°	N°	N°
1 (Gar)	<i>Myotis myotis/blythii</i>	204	74	140	204
2 (Vez)	<i>Myotis myotis/blythii</i>	86	0	70	86
3 (Vil)	<i>Myotis myotis/blythii</i>	59	0	50	59
4 (PDV)	<i>Myotis myotis/blythii</i>	19	0	14	19
5 (Brx)	<i>Myotis myotis/blythii</i>	57	0	40	57



# Laboratory analysis



## Active surveillance

- ✓ Lyssavirus: 18 positive sera
- ✓ Coronavirus: 1 alphaCoV
- ✓ Orthoreovirus: 6 MRVs

RFFIT EBLV1

PanCoV hnRT-PCR

VERO cells



Colony	Host species	LYSV		CoV		MRV	
		analyzed	positive	analyzed	positive	analyzed	positive
1 (Gar)	<i>Myotis myotis</i>	133/204	6	74/140	1	74/140	3
2 (Vez)	<i>Myotis myotis/M. blythii</i>	56/86	1	20/70		20/70	5
3 (Vil)	<i>Myotis myotis</i>	29/59	2	0/50		0/50	
4 (PDV)	<i>Myotis myotis</i>	8/19	0	0/14		0/14	
5 (Brx)	<i>Myotis myotis</i>	34/57	9	0/40		0/40	

### Full genome sequence (NGS)

- ✓ 2 strains: confirmed as **MRVs Type 1** (Id. 32129-2,6/2016)
- ✓ 4 strains: confirmed as **MRVs Type 2** (Id. 32129-1,3,7,8/2016)

*Genetic investigation of bat colonies is ongoing..*





# Introduction – Rhabdoviridae



## Rhabdoviridae

Phylogeny, genome organisations, known hosts and vectors

### Family Rhabdoviridae

#### Genera Lyssavirus

Vescivulovirus

Perhabdovirus

Sigmavirus

Ephemerovirus

Tibroviurus

Tupaiavirus

Sprivivirus

Cytorhabdovirus

Dichoravirus

Novirhabdovirus

Nucleorhabdovirus

Varicosavirus

Almendravirus

Sripuvirus

Ledantevirus

Hapavirus

Curiovirus

More than 130 viral species



**18 genera**

**ICTV approved**

<http://www.ictvonline.org/virusTaxonomy.asp>



# Results - Case description

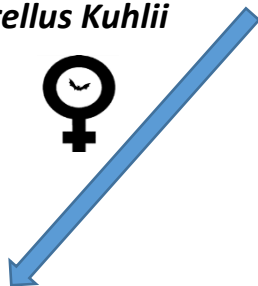


## Anamnesis and post-mortem findings

- Sensory depression
- Inappetence
- Normal body mass
- The **death** occurred three days after the admission to the center (October, 2016)



*Pipistrellus Kuhlii*



Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "B. Ubertini"



- **No pathological lesions indicative of infectious diseases** were observed at necropsy
- Injuries of patagium consistent with a cat bite

### Wildlife recovery center



Private citizen



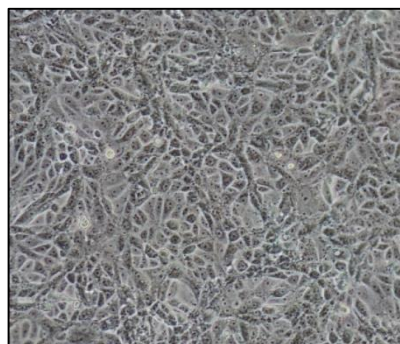
# Results - Cells culture



**CPE was observed on VERO cells inoculated with a pool of organs**



Cytophatic effect on infected VERO cells (2<sup>th</sup> pass, 3 dd pi)





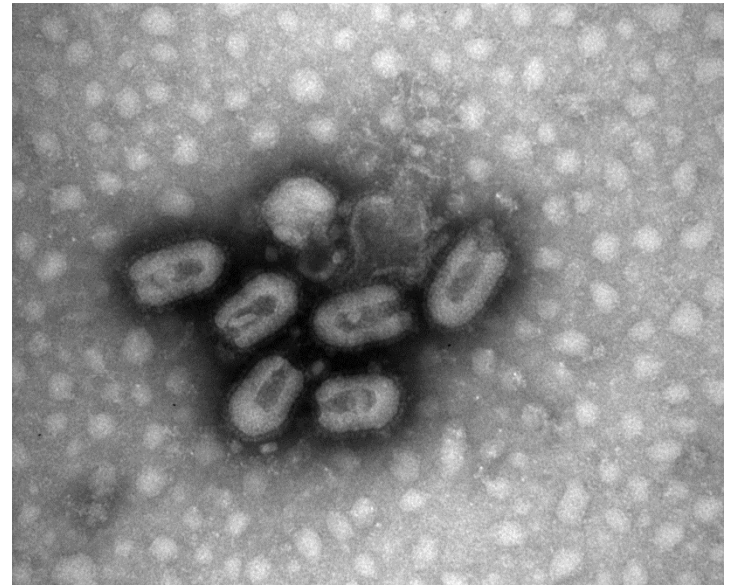


# Results - Virus identification



## Negative staining Electron Microscopy

- ✓ NsEM performed on cell supernatants revealed the presence of many distinct bullet-shaped viral particles referable to **rhabdovirus**.
- ✓ Tests aimed to **exclude positivity to rabies virus** and related lyssavirus virus resulted negative

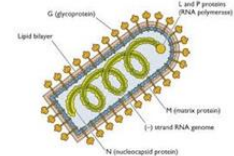




# Results - Molecular analysis



- The definitive assignment of the new isolate was confirmed by **NGS**.
- The **full genome sequence** of the new RHABV was determined starting from the cell culture supernatants with CPE
- The sequence was compared with those of strains downloaded from **GenBank**



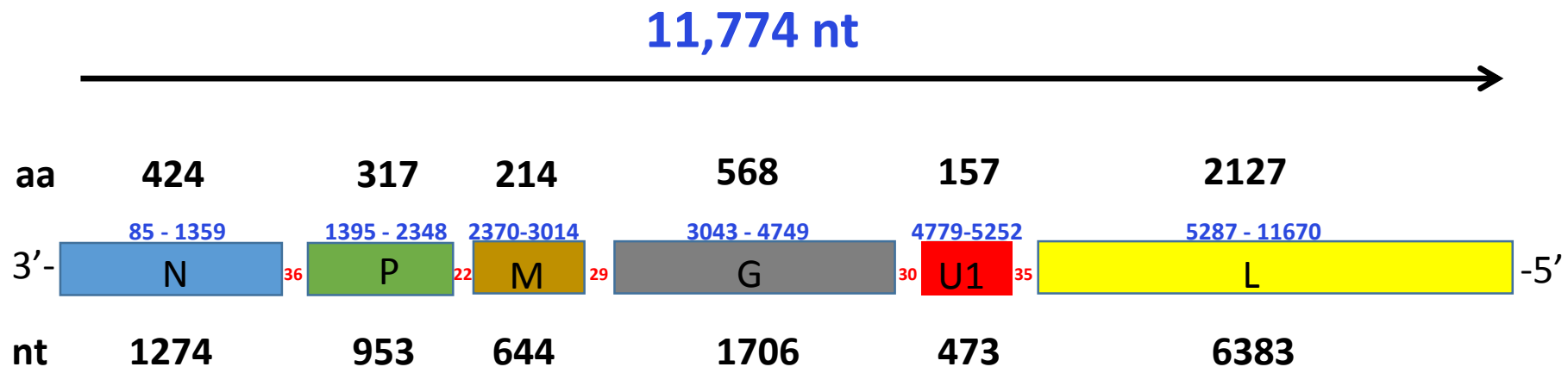


# Results – Molecular analysis



## Genome structure

- ✓ Complete genome size: 11,774 nt
- ✓ 5 genes encoding the canonical rhabdovirus structural proteins (N, P, M, G, L)
- ✓ additional transcriptional unit (U1) encoding a small protein (157 aa) between the G and L genes














# Results - Molecular analysis



## BLAST analysis. Highest amino acid sequence identities for each protein of the novel Vaprio virus (VAPV)

Protein	% similarity (query cover %)	RHABDO strain	Host	Country	GenBank Acession No.			
N	64 (100)	Le Dantec virus (LDV)	 Human (LDV)	1965, Senegal 	YP_009361868			
	63 (100)	Keuraliba virus (KEUV)			YP_009362195			
P	37 (99)	Keuraliba virus (KEUV)			 Rabbit (KEUV)	1968, Senegal 	YP_009362196	
	34 (99)	Le Dantec virus (LDV)					YP_009361869	
M	56 (98)	Le Dantec virus (LDV)						YP_009361870
	53 (98)	Keuraliba virus (KEUV)						YP_009362197
G	64 (86)	Le Dantec virus (LDV)				YP_009361871		
	61 (91)	Keuraliba virus (KEUV)				YP_009362198		
U1	30 (59)	Psychroflexus tropicus						WP_019037612
L	70 (99)	Keuraliba virus (KEUV)						AJR_28566
	69 (99)	Le Dantec virus (LDV)	AJR_28452					

Note: N, nucleoprotein ; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, large protein (RNA-dependent RNA polymerase)

## 11,774 nt

## 65% nucleotide identity to Le Dantec virus (LDV)

- LDV is the prototype strain of the putative genus Ledantevirus
- LDV is the only virus in the genus that has been associated with clinical disease in humans
- VAPV satisfy the species demarcation criteria of the ICTV, Rhabdoviridae Study Group for classification in the genus of Ledantevirus.

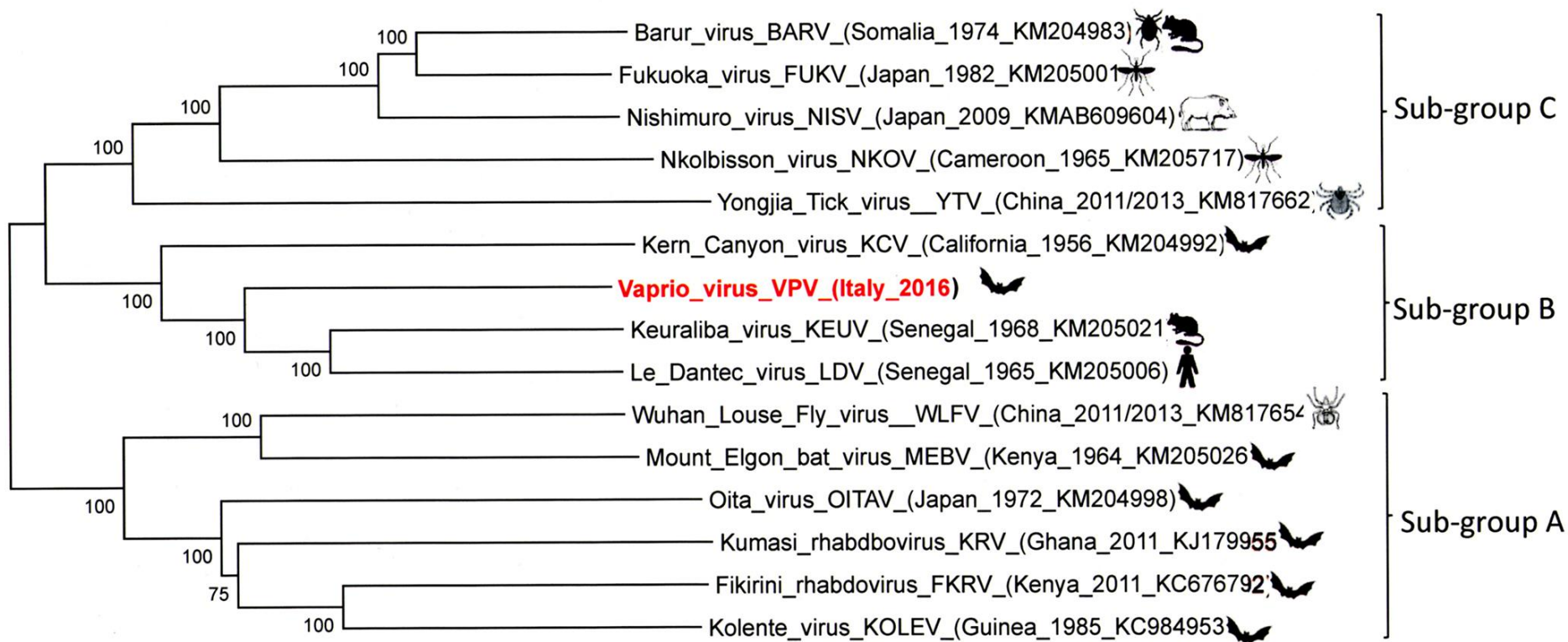


# Results - Phylogenetic analysis



## Genus **Ledantevirus**

**Phylogenetic tree based on the complete genome (11,774 nt) of the new Vaprio virus (VAPV) performed with all the 14 known viral species belonging to the genus Ledantevirus**



0.05

D. Lelli, Proserpi A, Moreno A, Chiapponi C, Gibellini AM, Debenedictis P, Leopardi S, Sozzi E, Lavazza A. Isolation of a novel Rhabdovirus from an insectivorous bat (*Pipistrellus kuhlii*) in Italy. VIRJ-D17-0064. *Submitted*



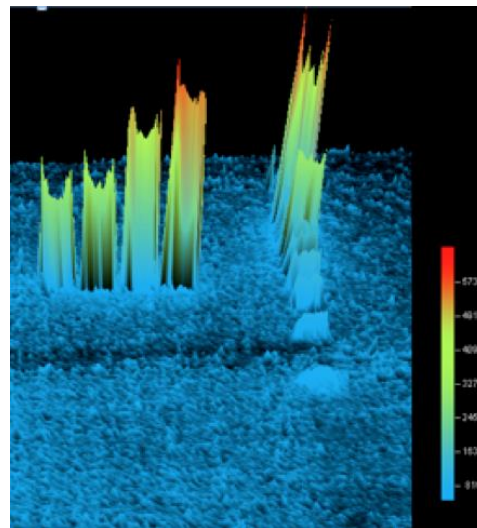
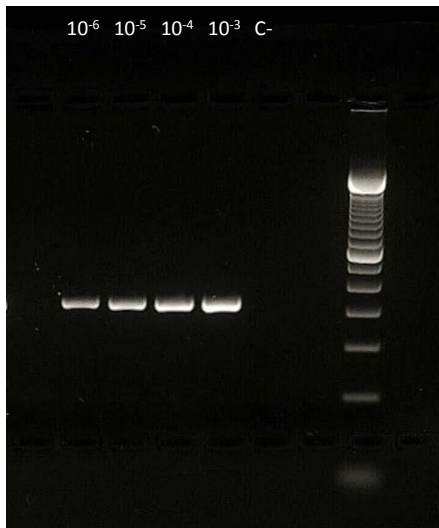
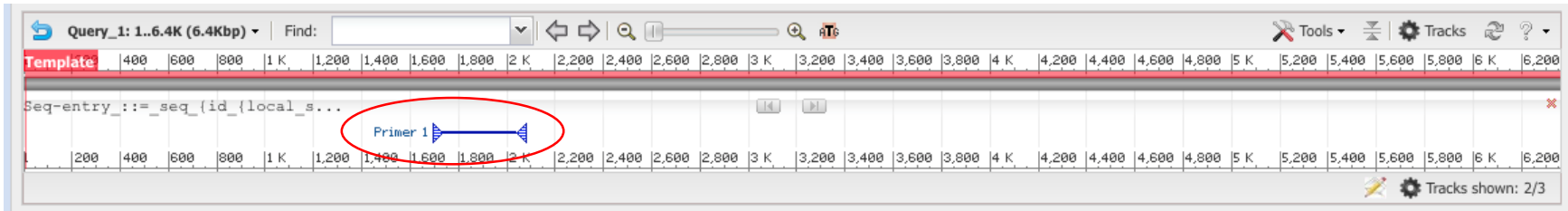
# Results – RT PCR



## One-Step end-point RT-PCR

✓ *NCBI-Primer Blast*

✓ *ORF 6 (L gene) – RDRP: 350 bp*



Specie	Nr.
<i>Hypsugo savii</i>	16
<i>Pipistrellus kuhlii</i>	6
<i>Plecotus auritus</i>	1
<b>Total</b>	<b>23</b>







# Discussion & Conclusions



- ✓ Insectivorous bats carry a wide variety of **CoVs** and **MRVs**
- ✓ Flavivirus.....
- ✓ Bats may play a key role in the generation of novel reassortant strains (e.g. MRVs) but also new/unknown viruses (e.g. VAPV) with unpredictable biological properties
- ✓ The Identification of novel viral agents in bat species such as *P. Khulii*, which is common in urban areas and known for its close interactions with humans, underlines the need for vigilance and stresses the importance to continue and improve virus surveillance in bats through the development and standardization of specific diagnostic tools
- ✓ This study enhances the cooperation between virologists and bat specialists and represents a synergic example of multidisciplinary approach to predict emergencies of public health interest.





# Acknowledgments



Thanks for your attention



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