Peer-reviewed version available at Viruses 2018, 10, 443; doi:10.3390/v10080443

Article

NEW ADENOVIRUS GROUPS IN WESTERN PALAEARCTIC BATS

Maria Iglesias-Caballero[1], Javier Juste[2], Sonia Vázquez-Morón[1,3], Ana Falcon[1], Carolina Aznar[1,3], Carlos Ibáñez[2], Francisco Pozo[1], Guillermo Ruiz[1], Jose M Berciano[1], Ignacio Garín[4], Jose Aihartza[4], Juan E Echevarría[1,3] , Inmaculada Casas[1*]

[1] Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Majadahonda-Pozuelo km 2. Majadahonda 28220, Madrid, Spain.
[2] Estación Biológica de Doñana, CSIC, Avda Américo Vespucio 16, 41092 Seville, Spain
[3] Centro de Investigación Biomédica de Epidemiología y Salud Pública, CIBERESP, Spain
[4] Department of Zoology and Animal Cell Biology, University of the Basque Country (UPV/EHU), Leioa 48940, Basque Country, Spain

Abstract: In the context of long-term screening for viruses on Western Palaearctic bats, we tested for the presence of adenovirus 1.392 oropharyngeal swabs and 325 stool samples taken from 27 bat species. Adenoviruses were detected in 12 species of the *Vespertilionidae* and the *Rhinolophidae* families. Fifty positive respiratory and 26 positive stool samples were studied. Phylogenetic analyses of partial hexon protein and partial DNA-dependent DNA polymerase genes, indicate all these bat adenoviruses belong to the genus *Mastadenovirus* but without constituting a monophyletic cluster. According to genetic identities, the new groups are distinct to the previously described *Bat mastadenovirus A* and *B* species, and contribute with potentially new members. Our data support that diversity of Bat mastadenovirus is host-dependent and increase the knowledge of potentially pathogenic virus from bats. For human concerns this knowledge is an important Public Health issue due to the active role of bats as viral reservoirs.

Keywords: Adenovirus, Western Palaearctic Bats, Phylogenetic analysis, Spain

1. Introduction

Bats are the second largest order of mammals, including more than 1,200 different species **[1]**. Their high vagility and the organization typically in social groups predispose them to infection and viral dissemination **[2]**. Extensive surveys have shown its susceptibility to host a wide range of viruses and the possibility to be a source of emerging infectious in humans **[3]**. The Order Chiroptera plays a role as a reservoir for many significant viruses such as Lyssavirus, Coronavirus, Herpesvirus, Filovirus, Reovirus, Paramyxovirus and Astrovirus, among others. Several studies have shown bats as a source of novel viruses, including Adenoviruses **[4–7]**.

Adenoviruses (AdVs) are subdivided in five genera, Mastadenovirus (mammals), Aviadenovirus (birds), Atadenovirus (mammals, birds and reptiles), Siadenovirus (poultry and amphibians) and Ichtadenovirus (fish) **[8]**. In 2008, the first AdV from a bat, Bat AdV1-FBV1, was isolated during attempts to establish a specific cell line from a Ryukyu flying fox (*Pteropus dasymallus yayeyamae*), in Japan **[9]**. Subsequently, after a search in 55 German free-ranging bats, family *Vespertilionidae*, a second, Bat AdV2 strain PPV1, was identified in 3 common pipistrelles (*Pipistrellus pipistrellus*), being the first AdV isolated from a microchiropteran bat and the second fully sequenced genome **[10]**, Bat mastadenovirus B. The first fully sequenced AdV genome from a bat was from a Rickett's

big-footed bat (*Myotis ricketti*), BtAdV3 strain TJM, named as Bat mastadenovirus A **[11]**. Several other studies have shown a large genetic viral diversity in bats from Brazil **[12]**, Japan **[9]**, Germany **[4,10,13,14]**, China **[11,15]**, Hungary **[5,14]**, Ghana **[16]**, Zambia **[17]**, Kenya **[7]** , South Africa **[18]** and USA **[19]**.

In Spain, rabies surveillance has become an important issue due to its geographic position between Africa and Europe **[20]**, particularly on bats with expected genetic flow between the South of Spain and the North of Morocco such as *Eptesicus isabellinus* **[21]**. Several studies confirmed both Iberian species of Eptesicus as rabies vectors **[22,23]** including the detection of the new Lleida bat lyssavirus **[24]**. Other studies have described new viruses, such as a novel Lloviu filovirus detected in dead *Miniopterus schreibersii* in the North of Spain **[25]**, 14 coronavirus distributed in new groups including 2 betacoronavirus related with the MERS-CoV group **[26]**, 42 potentially novel betaherpesvirus from the families *Vespertilionidae*, *Miniopteridae*, *Rhinolophidae*, *Molosidae* and *Pteropodidae* in the South and North of Spain **[27]**. These studies have increased the knowledge of new virus and their potential as human pathogens. Due to the active role of bats as viral reservoirs, this knowledge is an important part of the Public Health surveillance. Our study aimed to investigate the Bat AdVs groups and to describe their phylogenetic relations analyzing two distinct informative partial genes.

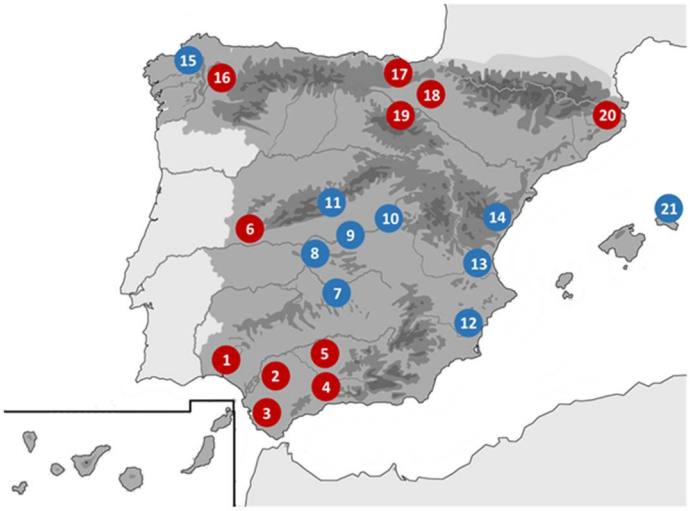
2. Materials and Methods

2.1. Origin of samples and preparation

During 2004 to 2016, in the context of rabies surveillance, a screening for other different virus was performed according to the General Research Program protocol of the Spanish Government (specific projects SAF2006-12784-C02, SAF2009-09172 and SAF2013-47194-P). Bats were captured and sampled in several campaigns across the Iberian Peninsula (Figure 1). Sampling methods followed the regulations and ethical procedures of the Spanish Bat Society (SECEMU). After being captured, each animal was identified, sexed, measured and weighed. For identification of cryptic species complexes, a wing-punch sample was taken for analysis of a cytochrome-b gene fragment **[21]**. For virological studies, oropharyngeal swabs (OPS) and stool samples (SS) were taken and homogenized in 1 ml of lysis buffer. After being studied and sampled, bats were released at the same location. Samples were sent to the Rabies National Reference Laboratory, aliquoted and stored at –80ºC until tested. Total nucleic acids were extracted from aliquots of 200 µl-buffered suspension and pellets were diluted in 50 µl of water **[28]**.

Figure 1: Geographical distribution of Bat capture locations in Spain. South of Spain: 1. Huelva, 2. Seville, 3. Cádiz, 4. Málaga, 5. Córdoba. Center of Spain: 6. Cáceres, 7. Ciudad Real, 8. Toledo, 9. Madrid, 10. Guadalajara, 11. Segovia, 12. Alicante, 13. Valencia, 14. Castellón. North of Spain: 15. A Coruña, 16. Lugo, 17. Vizcaya, 18. Navarra, 19. La Rioja, 20. Gerona. Balearic Islands: 21. Menorca. Red circles are locations with positive Bat AdVs.

Peer-reviewed version available at Viruses 2018, 10, 443; doi:10.3390/v10080443



2.2. Adenovirus detection by Generic PCR methods

Two independent generic PCR assays were used for the Adenoviridae family detection. For the screening of samples, panAdVHex nested PCR used previously for human AdVs genotyping, amplify one of the seven hypervariable regions of the hexon gene **[29,30]**. Five µl of nucleic acids extracted were added to 45 µl of reaction mixture containing 60 mM Tris-HCl (pH8.5), 15 mM(NH4)2SO4, 0.4 mM each of dNTPs (GE Healthcare, UK), 60 pmol of each primer and 2.5U AmpliTaq DNA Polymerase (Applied Biosystems, New Jersey USA). Temperature-time profiles were: 95°C-4 min and 40 cycles, 95°C-30 sec, 50°C-2 min, 72°C-30 sec. For nested reactions, same reagents and temperature-time profiles were used. Amplified products (±768 bp) were visualized by 2% agarose gel electrophoresis. To increase the phylogenetic accuracy, a panAdVPol hemi-nested PCR assay targeting a taxonomical informative fragment of the DNA-dependent DNA polymerase gene (DNApol) was designed and used. Five µl of extract was added to 20 µl of reaction mixture (LightCycler 480, Roche Diagnostics, Mannheim, Germany) and 10 pmol of the primers pol-F (5'GTIGCRAAIGAICCRTAGAGGGC 3') and pol-R (5'GTTTAYGAYATITGYGGMATGTAYGC 3'). Temperature-time profiles were: 95ºC-5 min, followed by 45 cycles, 95ºC-15 sec, 57ºC-2 min, 68ºC-30 sec. For heminested reactions, 2 µl of the previously amplified DNA and 10 pmol of the primers pol-F2 (5'AAIGAICCRTAGAGGGCRTTKGA 3') and pol-R were added to a reaction mixture containing 60 mM Tris-HCl (pH8.5), 15 mM(NH4)2SO4, 0.2 mM each of dNTPs, and 1.25U AmpliTaq DNA Polymerase. Temperature-time profiles were: 95ºC-5 min, followed by a two-step-cycle of 95ºC-15 sec and 62ºC-2 min 45 times. Amplified products (±450 bp) were visualized by 2 % agarose gel electrophoresis.

2.3. Sequence and phylogenetic analysis

Amplified products of the expected size were double-strand sequenced by Sanger chain-termination method using the BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The nucleotide sequences were compared with those published in GenBank database using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/) to assess and identify similar deposited AdV sequences. Two nucleotide multiple-sequence alignments from the hexon and DNApol genes, comprising a selection of available Mastadenovirus sequences from the GenBank database, were constructed using CLUSTAL X (v.2.0; http://www.clustal.org/). Phylogenetic analysis was performed with MEGA 5.2 software and were based on a Neighbor-Joining criterion using a Tamura 3 and Kimura 2-parameter models for the hexon and DNApol genes respectively, selected by Modeltest software **[31]**. Pairwise distance comparison between the predicted DNApol aminoacid sequences of Iberian Bat AdVs and Bat mastadenovirus A

and B was calculated using MEGA 5.2 software. Name for the putative new Bat AdVs was assigned using the bat host species abbreviation and the identification ring number.

3. Results

Bat species studied, year of capture, type of sample and the corresponding GenBank accession numbers for the Iberian Bat AdV sequences are listed in Table 1.

Table 1: Bat species studied, AdV positive results, year of capture and GenBank accession numbers. 1 Abb., Bat species abbreviations, 2 OPS, Oropharyngeal swabs, 3 SS, Stool samples, 4 Capture Year, 5 GenBank Accession number for hexon sequences, 6 GenBank Accession number for DNA polymerase sequences

Iberian Bat species			OPS[2]	SS[3]	Year[4]	Hexon sequences[5]	DNA-pol sequences[6]
Family	Name	Abb[1]					
Vespertilionidae	*Barbastella barbastellus*	Bba	0/38	0/4	07,08	N/A	N/A
	Eptesicus isabellinus	Eis	0	0/8	04,07	N/A	N/A
	Eptesicus serotinus	Ese	0	0/14	03,07	N/A	N/A
	Hypsugo savii	Hsa	0/31	3/26	07	HM856338,41,42	N/A
	Myotis alcathoe	Mal	0	0/1	07	N/A	N/A
	Myotis bechsteinii	Mbe	1/18	0/2	07	MF540611	N/A
	Myotis blythii	Mbl	0/29	0	04	N/A	N/A
	Myotis capaccinii	Mca	0/15	0	04,07	N/A	N/A
	Myotis daubentonii	Mda	0/63	0/41	04,07	N/A	N/A
	Myotis emarginatus	Mem	3/56	0	08	MF540608-10	N/A
	Myotis escalerai	Mes	0/13	0	04,07	N/A	N/A
	Myotis myotis	Mmy	1/79	0/1	04,07	HM856353	N/A
	Myotis mystacinus	Mmt	0/2	0/8	07	N/A	N/A
	Myotis nattereri	Mna	0/36	0/3	07	N/A	N/A
	Nyctalus noctula	Nno	3/122	0	07	MF540597-99	N/A
	Nyctalus lasiopterus	Nlas	10/139	6/40	07	HM856327-34,39-40,43, 50, MG132211	JX065117-20, 23,25-26,28
	Nyctalus leisleri	Nle	1/19	3/26	07	HM856344,51-52 HM86348	JX065124,27,29
	Pipistrellus kuhlii	Pku	12/350	2/4	07,16	MF540577-85,87,89	MF404970-73, MF404997,86
	Pipistrellus pipistrellus	Ppi	0/29	0/4	07,16	HM856349	
	Pipistrellus pygmaeus	Ppy	6/36	11/120	07,16	MF540575-76, 86,88,90-96	MF404968-69,74, 76-79,82-85,87-89
	Plecotus auritus	Pau	0/11	0/8	04,07	N/A	N/A
	Plecotus austriacus	Pas	0/10	0/6	04,07	N/A	N/A
Miniopteridae	*Miniopterus schreibersii*	Msc	0/152	0/2	04,07, 16	N/A	N/A
Rhinolophidae	*Rhinolophus euryale*	Reu	6/49	0	04,07, 08	MF540600-02,12-13 HM856335,MH521261	N/A
	Rhinolophus ferrumequinum	Rfe	7/90	1/3	04,07	MF540603-07,14	N/A

						HM856336-37	
	Rhinolophus hipposideros	Rhi	0/4	0/4	07,08	N/A	N/A
	Rhinolophus mehelyi	Rme	0/1	0	07,08	N/A	N/A
Total	27/32		50/1392	26/325		70 49OPS + 21SS	35 14OPS + 21SS

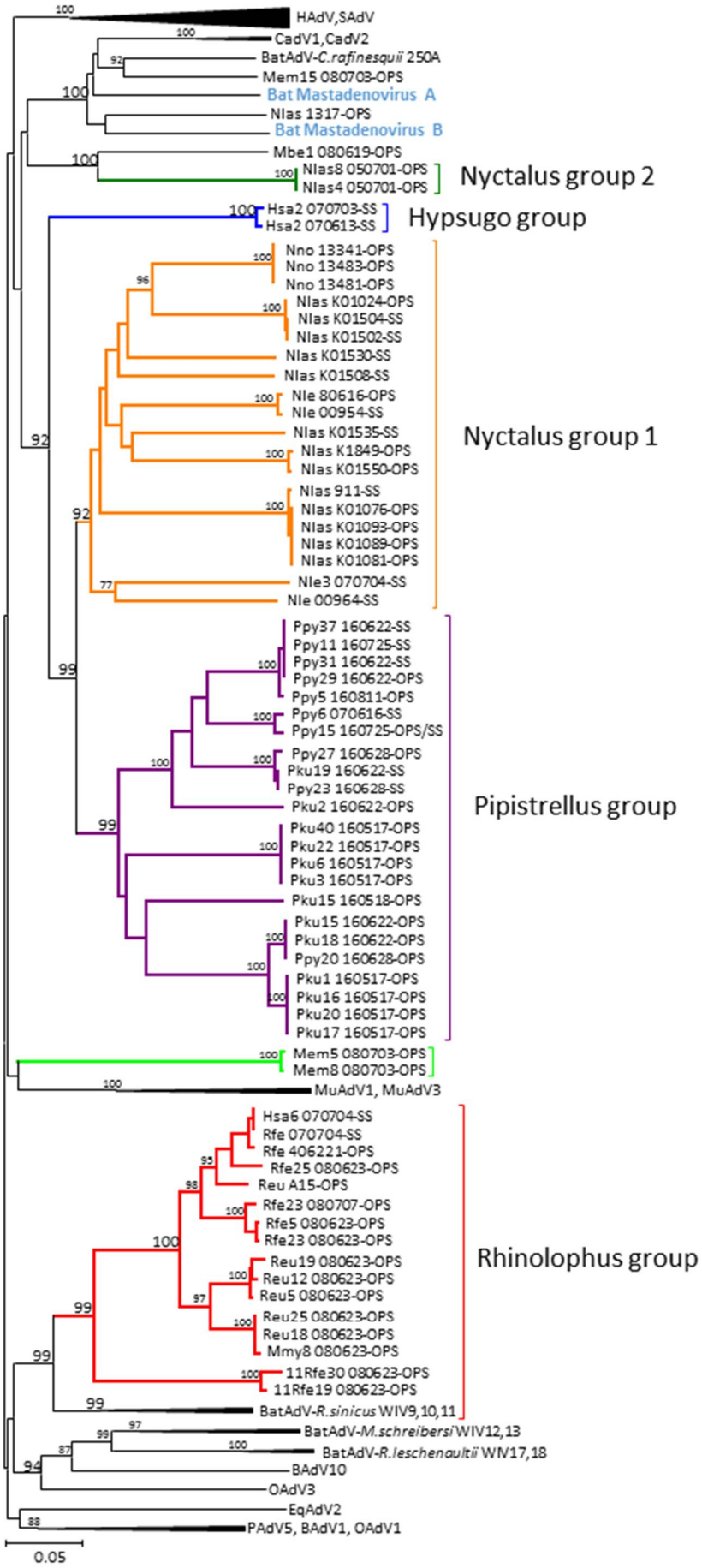
We studied for the presence of Bat AdV, a total of 1.717 samples, 1.392 OPS and 325 SS, representing 27 out of the 32 bat European species (http://secemu.org), belonging to the families *Vespertilionidae* (22 sspp), *Miniopteridae* (1 sp) and *Rhinolophidae* (4 sspp). Positive results were found in 50 OPS (3,6%) and in 26 SS (8,3%). Seventy different bats were positive and 3 bats were positive in both OPS and SS. Amplification of the partial AdV hexon gene was obtained in 70 samples, 49 OPS and 21 SS, and partial DNApol in 35 samples, 14 OPS and 21 SS. All amplified products were confirmed by sequencing and individual sequences were deposited in the GenBank database (Table 1). In 29 bats both genes were studied. Sequences of the hexon were obtained only in 41 and the DNApol only in 6 bats.

According to the geographical distribution of positive bats, Andalusia (South of Spain), was the region in which more positive bats were detected including several genera of the families *Vespertilionidae*, (*Pipistrellus*, *Myotis* and *Nyctalus*) and the *Rhinolophidae* (Rhinolophus). The majority of positive bats belonging to the *Pipistrellus* genus corresponded to those sampled in Andalusia. All 59 Bat AdVs found in the *Rhinolophus* genus came also from Andalusian bats even though out of 78 bats sampled in the Basque Country (North) no positives were found. The 3 Nyctalus species (*N. noctula*, *N. lasiopterus* and *N. leisleri*, 23 bats) and 2 of the 3 Pipistrellus (*P. kuhlii* and *P. pygmaeus*, 28 bats) contributed the most to the list of positives detected in OPS and SS. Two out of 4 species of the Rhinolophus genus (*R. euryale* and *R. ferrumequinum*, 14 bats) also contributed mainly and AdV were detected only in OPS.

3.1. Phylogenetic analysis of Bat AdV sequences

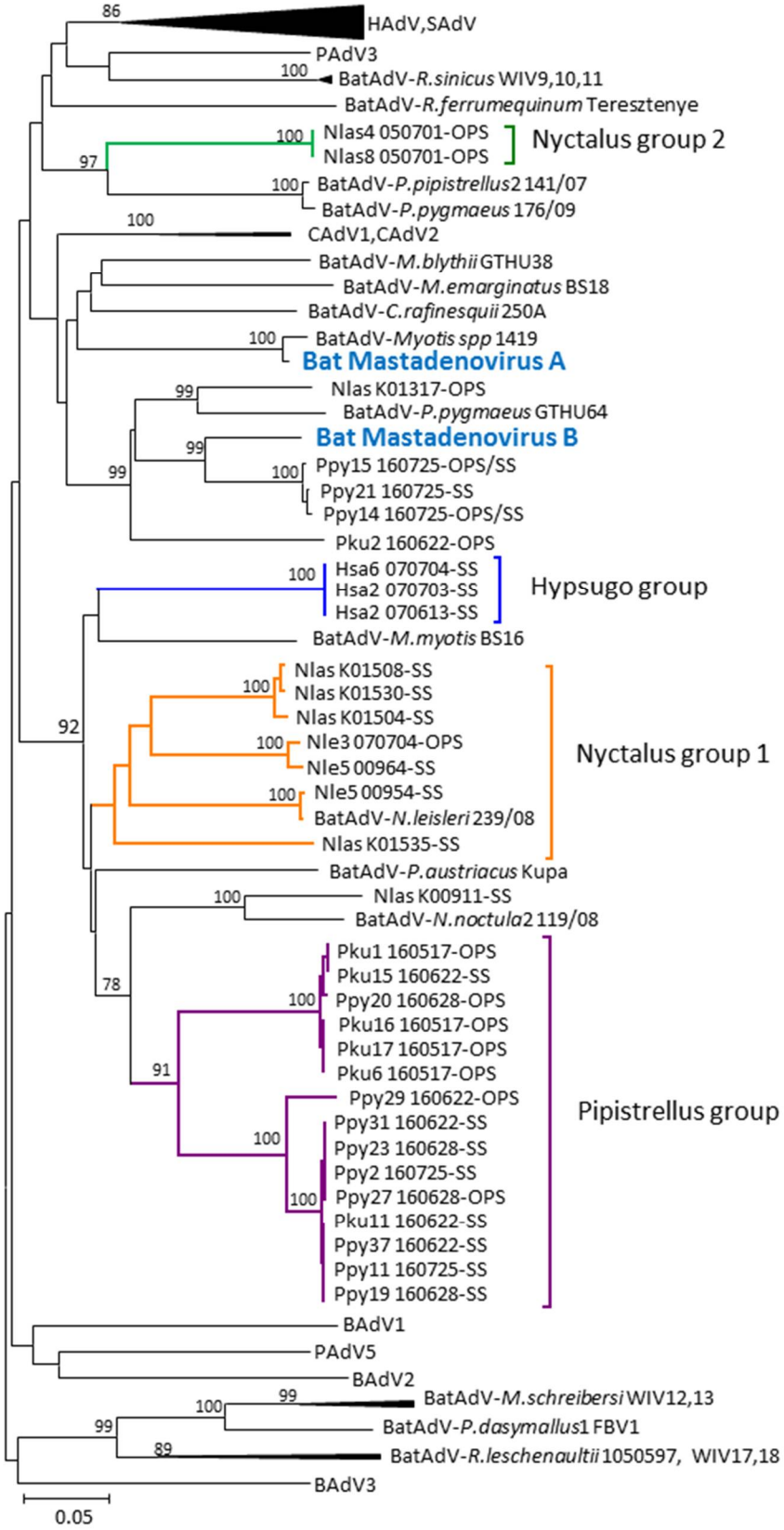
Our sequences from partial AdV hexon and DNApol genes, Figure 2 and Figure 3 respectively, were included within the genus *Mastadenovirus*. High bootstrap values supported clusters which differentiate the Bat mastadenovirus from the families *Rhinolophidae* and *Vespertilionidae*. Both genetic markers based on partial sequences reproduced the clustering obtained using the complete genome sequences with high bootstrap values **[18]**.

Figure 2: Phylogenetic tree based on the analysis of the hexon partial gene. Trees were estimated with MEGA 5.2 software by using the neighbor-joining method on Tamura 3 parameters model. A bootstrap test was replicated for 5000 times. Numbers represent percentage bootstrap support. GenBank accession numbers for the sequences included in the tree are as follows: Bat mastadenovirus A (GU226970), Bat mastadenovirus B (JN252129), human AdVs: type 1 (AF534906), type 2 (J01917), type 3 (DQ086466), type 4 (AY594254), D8 strain Ger/Berlin/04_2003 (KT862545), type 9 (AJ854486), type 12 (X73487), type 14 (FJ841902), type 16 (X74662), type 21 (KF528688), type 24 (JN226751), type 27 (JN226753), type 42 (JN226761), type 45 (JN226764), simian AdV: type 1 (AY771780), type 4 (KP853121), ovine AdV: type 1 (DQ630754), type 3 strain (DQ630756), porcine AdV 5 (AF289262.1), murine AdV: type 1 (M81889), type 3 (EU835513), bovine AdV: type 1 (DQ630761), type 10 (AF282774), canine AdV: type 1 (KX545420), type 2 (U77082), equine adenovirus type 2 (L80007), Bat mastadenovirus: *M . schreibersi* WIV12 (NC_030860), *M . schreibersi* WIV13 (NC_030874), *R. leschenaultii* WIV17 (NC_034626), *R. leschenaultii* WIV18 (NC_035072), *R. sinicus* WIV9 (NC_029898), *R. sinicus* WIV10 (NC_029899), *R. sinicus* WIV11 (NC_029902), *C. rafinesquii* 250-A (NC_031948)



163

Figure 3: Phylogenetic tree based on the analysis of the DNA-dependent DNA polymerase partial gene. Trees were estimated with MEGA 5.2 software by using the neighbor-joining method on Kimura 2 parameters model. A bootstrap test was replicated for 5000 times. Numbers represent percentage bootstrap support. GenBank accession numbers for the sequences included in the tree are as follows: Bat mastadenovirus A (GU226970), Bat mastadenovirus B (JN252129), human AdV: type 1 (AF534906), type 2 (J01917), type 3 (DQ086466), type 4 (AY594254), type 5 (AY339865), type 7 (AY594256), type 6 (HQ413315), type 9 (AJ854486), type 12 (X73487), type 17 (AF108105), type 19 (JQ326209), type 26 (EF153474), type 48 (EF153473), type 53 (AB605245), simian AdV: type 1 (AY771780), type 4 (KP853121), bovine AdV: type 2 (AF252854), type 3 (AF061654), bovine adenovirus A (AC_000191), porcine AdV: type 3 (AB026117), type 5 (AF289262), canine AdV: type 1 (KX545420), type 2 (U77082), Bat mastadenovirus: *M . schreibersi* WIV12 (NC_030860), *M . schreibersi* WIV13 (NC_030874), *R. leschenaultii* WIV17 (NC_034626), *R. leschenaultii* WIV18 (NC_035072), *R. sinicus* WIV9 (NC_029898), *R. sinicus* WIV10 (NC_029899), *R. sinicus* WIV11 (NC_029902), *C. rafinesquii* 250-A (NC_031948), *P. austriacus* Kupa (JN167523), *R. ferrumequinum* Teresztenye (JN167522), *Myotis spp 1419* (GU226962), *R. leschenaultia* 1050597 (HQ529709), *N. noctula2* 119/08 (KM043096), *M. emarginatus* BS18 (KM043084), *M. myotis* BS16 (KM043106), *M. blythii* GTHU38 (KM043086), *N. leisleri* 239/08 (KM043102), *P. pygmaeus* GTHU64 (KM043090), *P. pygmaeus* 176/09 (KM043091).



182

183

184

3.2. Partial AdV hexon gene sequence analysis

Only 2 of our 73 Bat AdVs were included in a significantly supported group clustered with both reference Bat mastadenovirus A (GU226970) and B (JN252129), detected in a *Myotis ricketti* from China **[11]** and in a *Pipistrellus pipistrellus* from Germany **[13]**, respectively. These 2 Bat AdVs were detected in OPS from a *Nyctalus lasiopterus*, Nylas_K01317 (HM856343), and from a *Myotis emarginatus*, Mem15_080703 (MF540610), highly related with a Bat mastadenovirus 250-A (KX871230) from a *Corynorhinus rafinesquii* captured in USA **[19]**.

The rest of the 71 Bat AdVs clustered in six groups with significantly bootstrap values, supporting potential novel groups within the genus *Mastadenovirus*. These new groups are host differentiated: Nyctalus group 1, Nyctalus group 2, Pipistrellus group and Hypsugo group of the *Vespertilionidae* family and a Rhinolophus group of the *Rhinolophidae* family (Figure 2 and 3).

Nyctalus group 1 clustered 13 AdVs from *N. lasiopterus*, 4 from *N. leisleri*, and 3 from *N. noctula*, highly associated with the Pipistrellus group. Nyctalus group 2 clustered apart including 2 AdVs from 2 distinct *N. lasiopterus* and 1 from a *Myotis bechsteinii* (Mbe1_080619 (MF540611). In a well-defined Pipistrellus group (bootstrap 99) cluster 13 AdVs from *P. kuhlii* and 9 from *P. pygmaeus*. Similarly, a well-defined group included sequences from Rhinolophus, 8 from *R. ferrumequinum*, and 6 from *R. euryale*, and 2 other from a *Myotis myotis* (Mmy-8_080623) and 1 from *Hypsugo savii* (Hsa6_070704). This cluster was highly supported and included 3 Bat AdVs detected in *R. sinicus* captured in China **[6]**. Furthermore, 2 distinct AdV detected from 2 *Hypsugo savii* bats were grouped in one independent cluster defined as Hypsugo group, highly related with the Nyctalus-group 1 and the Pipistrellus group. Two AdV detected in two *Myotis emarginatus* could defined a new Myotis group (Figure 2)

3.3. Partial AdV DNA-dependent DNA polymerase gene sequences

The groups defined in this gene were clearly associated by host with lower support in some nodes and less resolution comparing with the hexon partial gene analysed (Figure 3).

Five AdVs detected in *Pipistrellus pygmaeus* (Ppy14_160725 both OPS and SS, Ppy15_160725 both OPS and SS, Ppy21_160725) clustered together with the reference Bat mastadenovirus B (JN252129) in a group which included other 3 detected in 1 *Pipistrellus kuhlii* (Pku2_160622), in 1 *Nyctalus lasiopterus* (Nylas_K01317) and in 1 *P. pigmaeus* GTHU64 captured in Hungary **[14]**. This group, which included 6 AdVs found in the genus *Pipistrellus*, was separated from the rest of our Pipistrellus Bat AdVs.

Sequences from the genus *Nyctalus* were similar with those defined in the hexon gene with the exception of Nylas_K00911 detected in *a N. lasiopterus*, from the group 1 in the hexon gene, associated with an AdV detected in a *N. noctula* (KM043110) from Hungary. Nyctalus group 2 was clustered with 2 different detected in a *P. pipistrellus* (KM043096) and in a *P.pygmaeus* (KM43091) from Hungary. Five AdVs detected in *P. kuhlii* and 12 in *P. pygmaeus* clustered together defining a group as it occurs in the hexon gene. In the Hypsugo group, Hsa6_070704 detected in a *H. savii* clustered in the Hypsugo group unlike in the hexon gene. No positive results were obtained in this gene with the rhinolophid bats.

Pairwise distance matrix values obtained from the partial amino acid sequence of DNApol, supported the new groups (Table 2). According to the data, none of the Iberian Bat AdVs were related with the reference Bat mastadenovirus A exceeding the pairwise distance (>15 %). Otherwise, 3 AdV detected in *Pipistrellus pygmaeus* (Ppy14_160725, Ppy15_160725 and Ppy21_160725) were very similar to the reference Bat mastadenovirus B.

Table 2: Spanish Bat mastadenoviruses classified by the aminoacid distance matrix analysis based on partial DNA-dependent DNA polymerase. [1]Values more than 15% are potentially new species following the ICTV demarcation

Group of Bat AdV	Tentative virus name	% aa pairwise distances[1]	
		BatAdV A	BatAdVB
Bat AdVs associated with Bat AdVB	Bat mastadenovirus *P.pygmaeus* 14 160725	31,4	11,8
	Bat mastadenovirus *P.pygmaeus* 15 160725	31,4	12,7
	Bat mastadenovirus *P.pygmaeus* 21 160725	32	12,2
Potentially novel Bat AdVs	Bat mastadenovirus N. lasiopterus K01317	33,8	25,5
	Bat mastadenovirus *N. lasiopterus* K01508	35	41,8
	Bat mastadenovirus N. lasiopterus K01530	35,7	42,5
	Bat mastadenovirus N. lasiopterus K01504	35,7	42,5
	Bat mastadenovirus N.leisleri 3-070704	32,5	42,5
	Bat mastadenovirus *N.leisleri* 5-00964	34,5	44
	Bat mastadenovirus *N.leisleri* 5-00954	39,7	41,8
	Bat mastadenovirus *N. lasiopterus* K01535	41,8	52,1
	Bat mastadenovirus *N. lasiopterus* K00911	43,5	46,1
	Bat mastadenovirus *N. lasiopterus* 4- 050701	42,6	38,8
	Bat mastadenovirus *N. lasiopterus* 8- 050701	42,6	38,8
	Bat mastadenovirus P.kuhlii 2 160622	37,5	23,8
	Bat mastadenovirus *P.kuhlii* 1 160517	39,4	39
	Bat mastadenovirus *P.kuhlii* 15 160622	39,4	39
	Bat mastadenovirus *P.pygmaeus* 20 160628	38,7	39
	Bat mastadenovirus *P.kuhlii* 16 160517	38,7	38,3
	Bat mastadenovirus *P.kuhlii* 17 160517	38,7	38,3
	Bat mastadenovirus *P.kuhlii* 6 160517	38,7	38,3
	Bat mastadenovirus *P.pygmaeus* 29 160622	44,8	36,5
	Bat mastadenovirus P.pygmaeus 31 160622	41,2	41,6
	Bat mastadenovirus *P.pygmaeus* 23 160628	41,2	41,6
	Bat mastadenovirus *P.pygmaeus* 2 160725	41,2	41,6
	Bat mastadenovirus *P.pygmaeus* 27 160628	41,2	41,6
	Bat mastadenovirus *P.kuhlii* 11 160622	42	40,9
	Bat mastadenovirus *P.pygmaeus* 37 160622	42	40,9
	Bat mastadenovirus *P.pygmaeus* 11 160725	42	40,9
	Bat mastadenovirus P.pygmaeus 19 160628	42	40,9
	Bat mastadenovirus *H. savii* 6 070704	41	41,7
	Bat mastadenovirus *H. savii* 2 070613	41	41,7
	Bat mastadenovirus *H. savii* 2 070703	41	41,7

4. Discussion

In this work we describe the detection and the phylogenetic relationships among potentially new Bat mastadenovirus and known AdVs from bats using two different gene markers. Our study shows, for the first time, their diversity in bats captured in the South of Europe and particularly in our country which reveals a crucial importance for its strategic geographical placement, as a corridor between Africa and Europe.

Previous studies have shown a high diversity of AdVs found in bat species analyzed across Europe, Asia and Africa **[9,12,13,15,17,19]**. To further study of AdV in bats, 27 out of the 32 Iberian bat species were examined obtaining positive results in 12 species framed in 6 bat genera. In Centre of Europe, Hungary and Germany, have also found positive results for AdVs in 9 of these 12 species **[14]**. With the aim of having a broad representation of the AdV diversity in the Iberian bats, a total of 1.717 biological samples were analysed being the biggest AdV screening in bats ever studied. These bats were captured within Spain in a variety of habitats, from the Pyrenees and Cantabrian mountain ranges in the North to the Mediterranean South considered as natural border with Africa, and including several bat species with possible gene flow across the Gibraltar Strait **[32]**.

The percentage of AdV positive bats was 3,6 % in OPS and 8,3 % in SS over the 18,6% in German samples and the 9,9% in Hungary **[14]**. These marked differences could be explained by bat health conditions and/or the use of different type of biological samples, from the homogenised internal organ tissues taken in dead or injured bats in the German study to healthy bats and guano samples in roosting places in the Hungarian. Positive Bat AdV percentages similarity between our study and the Hungarian could be explained by the type of samples studied (OPS and SS). It is noteworthy the absence of AdVs in some bats such as the bent-winged *Miniopterus schreibersii*, despite the large number of individuals of this species screened. Similar negative results were found in Germany and Hungary **[14]**. Most of the AdV positive bats were found within the diverse bat family *Vespertilionidae*, and particularly within the tribe *Pipistrellini* (*Pipistrellus* and *Nyctalus*), whereas they seem absent from other bat tribe *Plecotinii* (*Barbastella* and *Plecotus*). Within the subfamily *Myotinae*, bats were found positive in several species, although sparsely along the phylogenetic trees without making any monophyletic cluster. Interestingly, AdVs were not found in some Myotis, *M. daubentonii*, despite it was well represented in the screening (n=60 and n=41 for OPS and SS, respectively).

Previous studies mostly focused on the analysis of guano and internal tissues **[9,12–14,17]**. The analysis of OPS for the screening of AdV is a novelty of this study that has allowed AdV detection in the upper respiratory tract of bats and the reconnaissance of a possible faecal-oral transmission substantiate with positive results in both type of samples, such as in two *P. pygmaeus* (Ppy15_160725 and Ppy14_160725) with the same Bat AdV in OPS and SS. The phylogenetic reconstructions identify, in both type of samples, AdVs highly related in different groups of bats, supporting this possible oral-faecal transmission. An important reason for the study of OPS in bats is the fact that many human AdV serotypes have not a specific well identified cellular receptor, and given that replicate poorly in animals **[33]**, the understanding of factors that define tropism and transmission during a natural infection increase the knowledge of AdV infections, especially in bats that are now considered as emerging and re-emerging infectious diseases vectors.

Previous authors have published new Bat mastadenovirus mostly based on the phylogenetic analysis of a short and informative fragment of the DNApol gene **[10,11,13,14]**. This is a well preserved gene involved in viral transcription **[34]**. Despite its extensive use in phylogenetic analysis of new human and animal AdVs, the resolution of the phylogenetic reconstruction based on it is limited (less than 100 aminoacids). The PCR presented in this study amplified ±450 bp, offering the possibility to increase the resolution in the correspondent phylogenetic tree. However, with the aim to compare our sequences with the previously published from the Centre of Europe **[14]** and the

reference available in the GenBank database, the length was reduced to 277 bp. Currently, ICTV accepted two Bat AdVs, Bat mastadenovirus A **[11]** and Bat mastadenovirus B **[13]**. According to the taxonomic criteria **[8]** and based on the analysis of distance matrix, the Bat mastadenoviruses presented in our study are potentially new for the genus Mastadenovirus and very divergent from the ICTV references with the exception of three detected in *P.pygmaeus* (Ppy15_160725, Ppy21_160725, Ppy14_160725). Moreover, one *P. kuhlii* (Pku2_160622) and one *N. lasiopterus* (Nylas_K01317) were associated with the Bat mastadenovirus B although the amino acid pairwise distance exceeded the 15% of difference suggesting potentially a new Bat AdVs. It is remarkable that Bat AdVs obtained from the species *P. kuhlii* and *P. pygmaeus* clustered together in two well supported groups indicating host specificity even at the species level.

In this work, the identification of new Bat AdVs is further supported by the results obtained using the hexon gene a more variable protein **[10,11,16,19]** which contains seven hypervariable regions identified as viral epitopes **[35]**. Nucleic acids variation define the different human serotypes **[36]**. Our generic PCR in the hexon gene was designed out the hypervariable region 7 and the analysis of the sequences obtained has proved the concordance between genotype and serotype in human AdVs **[29]**.

The evolutionary relationships based on the two genetic markers are presented separately since they provide different information according to their different mutation rates. Both genes agree in the main structure of their tree topologies and clusters, and both provide support for a presumably new Iberian Bat mastadenoviruses clustering and distinguishing between the families *Vespertilionidae* and *Rhinolophidae* in the phylogenies. Most of the available AdVs in the Genbank database grouped by host bats within the three monophyletic groups corresponding to their bats hosts genera *Pipistrellus*, *Nyctalus* and *Rhinolophus*. This relationship is also supported by the phylogenetic analysis of the DNApol gene in which the AdV detected in a *N. leisleri* (Nyle_00954) clusters with a Bat AdV detected in a *N. leisleri* sampled in Hungary **[14]**. In our sampling, more basal relationships among the main bat hosts are much harder to be traced back due to the lack of representation of important bat groups such as *Scotophillinii*, *Nycticeinii* and *Plecotinii* within the family *Vespertilionidae*. These basal relationships were clearly recovered from the widespread Herpesviruses **[27]** but still, AdVs could represent another example of parallel evolution of DNA virus and their bat hosts. The phylogenetic analysis of partial hexon gene proved that there are not differences between AdVs from bats captured in the South and the North of Spain, as it is shown in a *P. pygmaeus* (Pyi6_070616) collected in Lugo (North), and the *P. pygmaeus* (Ppy15_160725) collected in Seville (South).

Although most of the AdVs cluster by their bat host, there are also some exceptions. In the hexon gene, first the AdV detected in a *M. myotis* (Mmy8_080623) clusters with the group composed of two different species of Rhinolophus bats. It is well known that many Myotis colonies share roosts with several species of the genus *Rhinolophus* and this could be the origin of the inter-specific transmission between these two bat species. Second, the AdV detected in a *H. savii* (Hsa6_070704), clusters again within those detected from the Rhinolophus group despite the DNApol gene reveals a specific AdV group in three different *H. savii*. In this second example, a natural transmission seems less likely since the two species have very different life history and barely share any ecological requirement. Nevertheless, the description of recombinant viruses is a common phenomenon in human AdV **[37]**, and could explain the different results, depending on the genetic marker used. However, this possible recombination in Bat AdVs requires a further confirmation by the complete genomic sequence. A third exception shows the AdV detected in a *M. emarginatus* (Mem15-080703) that cluster together with a Bat AdV detected in a *Corynorhinus* rafinesquii and two others detected in Myotis bats from Hungary. The *C. rafinesqii* is a vespertilionid bat which distribution is restricted to the Southeast of North America and Mexico **[19,38]**. The connexion between these viruses is a puzzle given that their hosts are far apart geographically and evolutionary, although it could be related to a recent colonization of North America by Palearctic Myotis **[39]**.

In conclusion, based on the analysis of two different genetic markers used to study two different type of samples, the present study contributes with potentially new members from Mastadenovirus genus distinct to the previously reference described Bat mastadenovirus A y B **[11,13]**. The new AdV groups were detected in bats captured in a broad geographical region and generate data supporting that diversity of Bat mastadenovirus is associated by host and the distribution of the host.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Tentative viruses names, type of sample and localization of *Pipistrellus* group, Table S2: Tentative viruses names, type of sample and localization of *Nyctalus* group, Table S3: Tentative viruses names, type of sample and localization of *Hypsugo* and *Myotis* groups and Table S4: Tentative viruses names, type of sample and localization of *Rhinolophus* group.

Author Contributions: Conceptualization, IC,JJ,CI and JEE.; Methodology, MIC and IC; Validation, IC and MIC; Formal Analysis, MIC, IC, JJ, CI, AF and GR; Investigation, MIC,IC,AF and GR.; Resources, JJ,CI,JMB,IG and JA.; Data Curation, MIC, SVM, CAL and IC.; Writing-Original Draft Preparation, MIC and IC.; Writing-Review & Editing, MIC,IC and JJ.; Supervision, IC, JJ and JEE.; Project Administration, JEE, SVM and JJ; Funding Acquisition, JEE

Funding: This project was financially supported by an agreement between the Public Health Department of the Spanish Ministry of Health and the Instituto de Salud Carlos III for the development of "Rabies Surveillance in Spain" and by projects SAF 2006-12784-C02-01 and SAF 2006-12784-C02-02 of the General Research Programme of the Spanish Ministry of Science and Education

Acknowledgments: We thank the Genomics Unit of the Instituto de Salud Carlos III for the sequencing of amplified products. We thank all members of the Spanish Bat Conservation Society (SECEMU) that generously contributed with their samples to their present study.

Conflicts of Interest: None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

References

1. IUCN SSC Bat Specialist Group Available online: http://www.iucnbsg.org/ (accessed on May 14, 2018).
2. Calisher CH; Childs JE; Field HE; Holmes KV; Schountz T Bats: important reservoir hosts of emerging viruses. *Clin. Microbiol. Rev.* **2006**, *19(3):531-45*, doi:10.1128/CMR.00017-06.
3. Wong S; Lau S; Woo P; Yuen KY Bats as a continuing source of emerging infections in humans. *Rev Med Virol* **2007**, doi:10.1002/rmv.520.
4. Drexler JF; Corman VM; Wegner T; Tateno AF; Zerbinati RM; Gloza-Rausch F; Seebens A; Müller MA; Drosten C Amplification of emerging viruses in a bat colony. *Emerg. Infect. Dis.* **2011**, *17(3):449-56*, doi:10.3201/eid1703.100526.
5. Jánoska M; Vidovszky M; Molnár V; Liptovszky M; Harrach B; Benkö M Novel adenoviruses and herpesviruses detected in bats. *Vet. J.* **2011**, *189(1):118–21*, doi:10.1016/j.tvjl.2010.06.020.
6. Tan, B.; Yang, X.-L.; Ge, X.-Y.; Peng, C.; Zhang, Y.-Z.; Zhang, L.-B.; Shi, Z.-L. Novel bat adenoviruses with an extremely large E3 gene. *J. Gen. Virol.* **2016**, *97*, 1625–1635, doi:10.1099/jgv.0.000470.
7. Waruhiu C; Ommeh S; Obanda V; Agwanda B; Gakuya F; Ge XY; Yang XL; Wu LJ; Zohaib A; Hu B; Shi ZL Molecular detection of viruses in Kenyan bats and discovery of novel astroviruses, caliciviruses and rotaviruses. *Virol. Sin.* **2017**, *32(2):101-114*, doi:10.1007/s12250-016-3930-2.
8. Harrach B; Benkö M; Both G; Brown M; Davison AJ; Echavarría M; Hess M; Jones MS; Kajon A; Lehmkuhl AD; Mautner V; Mittal SK; Wadell G Family Adenoviridae. In *Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth report of the International Committee of Taxonomy of Viruses.*; Elsevier: San Diego, USA, 2011; pp. 125–141.

9. Maeda K; Hondo E; Terakawa J; Kiso Y; Nakaichi N; Endoh D; Sakai K; Morikawa S; Mizutani T Isolation of novel adenovirus from fruit bat (Pteropus dasymallus yayeyamae). *Emerg. Infect. Dis.* **2008**, *14(2):347–9.*, doi:10.3201/eid1402.070932.
10. Kohl C; Vidovszky M; Mühldorfer K; Dabrowski PW; Radonic A; Nitsche A; Wibbelt G; Kurth A; Harrach B Genome analysis of bat adenovirus 2: indications of interspecies transmission. *J. Virol.* **2012**, *86:1888-92*.
11. Li Y; Ge X; Zhang H; Zhou P; Zhu Y; Zhang Y; Yuan J; Wang LF; Shi Z Host range, prevalence, and genetic diversity of adenoviruses in bats. *J. Virol.* **2010**, *84(8):3889-97*, doi:10.1128/JVI.02497-09.
12. Lima FE; Cibulski SP; Elesbao F; Carnieli Junior P; Batista HB; Roehe PM; Franco AC First detection of adenovirus in the vampire bat (Desmodus rotundus) in Brazil. *Virus Genes* **2013**, *47(2):378-81*, doi:10.1007/s11262-013-0947-6.
13. Sonntag M; Mühldorfer K; Speck S; Wibbelt G; Kurth A New adenovirus in bats, Germany. *Emerg. Infect. Dis.* **2009**, *15(12):2052–5*, doi:10.3201/eid1512.090646.
14. Vidovszky M; Kohl C; Boldogh S; Görföl T; Wibbelt G; Kurth A; Harrach B Random sampling of the Central European bat fauna reveals the existence of numerous hitherto unknown adenoviruses. *Acta Vet. Hung.* **2015**, *63(4)*, 508–25.
15. Tan B; Yang XL; Ge XY; Peng C; Liu HZ; Zhang YZ; Zhang LB; Shi ZL Novel bat adenoviruses with low G+C content shed new light on the evolution of adenoviruses. *J. Gen. Virol.* **2017**, *98(4):739-748*, doi:10.1099/jgv.0.000739.
16. Baker, K. S.; Leggett, R. M.; Bexfield, N. H.; Alston, M.; Daly, G.; Todd, S.; Tachedjian, M.; Holmes, C. E. G.; Crameri, S.; Wang, L.-F.; Heeney, J. L.; Suu-Ire, R.; Kellam, P.; Cunningham, A. A.; Wood, J. L. N.; Caccamo, M.; Murcia, P. R. Metagenomic study of the viruses of African straw-coloured fruit bats: Detection of a chiropteran poxvirus and isolation of a novel adenovirus. *Virology* **2013**, *441*, 95–106, doi:10.1016/j.virol.2013.03.014.
17. Ogawa, H.; Kajihara, M.; Nao, N.; Shigeno, A.; Fujikura, D.; Hang'ombe, B. M.; Mweene, A. S.; Mutemwa, A.; Squarre, D.; Yamada, M.; Higashi, H.; Sawa, H.; Takada, A. Characterization of a Novel Bat Adenovirus Isolated from Straw-Colored Fruit Bat (Eidolon helvum). *Viruses* **2017**, *9*, 371, doi:10.3390/v9120371.
18. Vuren, P. J. van; Allam, M.; Wiley, M. R.; Ismail, A.; Storm, N.; Birkhead, M.; Markotter, W.; Palacios, G.; Paweska, J. T. A novel adenovirus isolated from the Egyptian fruit bat in South Africa is closely related to recent isolates from China. *Sci. Rep.* **2018**, *8*, 9584, doi:10.1038/s41598-018-27836-w.
19. Hackenbrack, N.; Rogers, M. B.; Ashley, R. E.; Keel, M. K.; Kubiski, S. V.; Bryan, J. A.; Ghedin, E.; Holmes, E. C.; Hafenstein, S. L.; Allison, A. B. Evolution and Cryo-electron Microscopy Capsid Structure of a North American Bat Adenovirus and Its Relationship to Other Mastadenoviruses. *J. Virol.* **2017**, *91*, doi:10.1128/JVI.01504-16.
20. Mingo-Casas P; Sandonís V; Vázquez-Morón S; Berciano JM; Juste J; Echevarría JE Rabies in Spain. A Peculiarity in Eurasia. *Ann. Virol. Res.* **2017**, *3(2): 1030.*
21. Juste, J.; Bilgin, R.; Muñoz, J.; Ibáñez, C. Mitochondrial DNA signatures at different spatial scales: from the effects of the Straits of Gibraltar to population structure in the meridional serotine bat (Eptesicus isabellinus). *Heredity* **2009**, *103*, 178–187, doi:10.1038/hdy.2009.47.
22. Vázquez-Morón, S.; Juste, J.; Ibáñez, C.; Berciano, J. M.; Echevarría, J. E. Phylogeny of European Bat Lyssavirus 1 in Eptesicus isabellinus Bats, Spain. *Emerg. Infect. Dis.* **2011**, *17*, 520–523, doi:10.3201/eid1703100894.

23. Vázquez-Morón, S.; Juste, J.; Ibáñez, C.; Ruiz-Villamor, E.; Avellón, A.; Vera, M.; Echevarría, J. E. Endemic Circulation of European Bat Lyssavirus Type 1 in Serotine Bats, Spain. *Emerg. Infect. Dis.* **2008**, *14*, 1263–1266, doi:10.3201/1408.080068.

24. Ceballos, N. A.; Morón, S. V.; Berciano, J. M.; Nicolás, O.; López, C. A.; Juste, J.; Nevado, C. R.; Setién, Á. A.; Echevarría, J. E. Novel Lyssavirus in Bat, Spain. *Emerg. Infect. Dis.* **2013**, *19*, 793–795, doi:10.3201/eid1905.121071.

25. Negredo, A.; Palacios, G.; Vázquez-Morón, S.; González, F.; Dopazo, H.; Molero, F.; Juste, J.; Quetglas, J.; Savji, N.; de la Cruz Martínez, M.; Herrera, J. E.; Pizarro, M.; Hutchison, S. K.; Echevarría, J. E.; Lipkin, W. I.; Tenorio, A. Discovery of an ebolavirus-like filovirus in europe. *PLoS Pathog.* **2011**, *7*, e1002304, doi:10.1371/journal.ppat.1002304.

26. Falcón A; Vázquez-Morón S; Casas I; Aznar C; Ruiz G; Pozo F; Perez-Breña P; Juste J; Ibáñez C; Garin I; Aihartza J; Echevarría JE Detection of alpha and betacoronaviruses in multiple Iberian bat species. *Arch. Virol.* **2011**, *156(10):1883–90*, doi:10.1007/s00705-011-1057-1.

27. Pozo F; Juste J; Vázquez-Morón S; Aznar-López C; Ibáñez C; Garin I; Aihartza J; Casas I; Tenorio A; Echevarría JE Identification of Novel Betaherpesviruses in Iberian Bats Reveals Parallel Evolution. *PLoS One* **2016**, *11(12):e0169153*, doi:10.1371/journal.pone.0169153.

28. Casas I; Powell L; Klapper PE; Cleator GM New method for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction assay. *J. Virol. Methods* **1995**, *53(1):25–36*.

29. Calvo, C.; García-García, M. L.; Sanchez-Dehesa, R.; Román, C.; Tabares, A.; Pozo, F.; Casas, I. Eight Year Prospective Study of Adenoviruses Infections in Hospitalized Children. Comparison with Other Respiratory Viruses. *PloS One* **2015**, *10*, e0132162, doi:10.1371/journal.pone.0132162.

30. Casas, I.; Avellon, A.; Mosquera, M.; Jabado, O.; Echevarria, J. E.; Campos, R. H.; Rewers, M.; Perez-Breña, P.; Lipkin, W. I.; Palacios, G. Molecular Identification of Adenoviruses in Clinical Samples by Analyzing a Partial Hexon Genomic Region. *J. Clin. Microbiol.* **2005**, *43*, 6176–6182, doi:10.1128/JCM.43.12.6176-6182.2005.

31. Posada, D.; Crandall, K. A. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **1998**, *14*, 817–818, doi:10.1093/bioinformatics/14.9.817.

32. García-Mudarra, J. L.; Ibáñez, C.; Juste, J. The Straits of Gibraltar: barrier or bridge to Ibero-Moroccan bat diversity? *Biol. J. Linn. Soc.* **2009**, *96*, 434–450, doi:10.1111/j.1095-8312.2008.01128.x.

33. Zhang, Y.; Bergelson, J. M. Adenovirus Receptors. *J. Virol.* **2005**, *79*, 12125–12131, doi:10.1128/JVI.79.19.12125-12131.2005.

34. Choi, K. H. Viral Polymerases. *Adv. Exp. Med. Biol.* **2012**, *726*, 267–304, doi:10.1007/978-1-4614-0980-9_12.

35. Roberts DM; Nanda A; Havenga MJ; Abbink P; Lynch DM; Ewald BA; Liu J; Thorner AR; Swanson PE; Gorgone DA; Lifton MA; Lemckert AA; Holterman L; Chen B; Dilraj A; Carville A; Mansfield KG; Goudsmit J; Barouch DH Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* **2006**, *441(7090):239–43*, doi:10.1038/nature04721.

36. Rux, J. J.; Kuser, P. R.; Burnett, R. M. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J. Virol.* **2003**, *77*, 9553–9566.

37. Kajon, A. E.; Dickson, L. M.; Murtagh, P.; Viale, D.; Carballal, G.; Echavarria, M. Molecular Characterization of an Adenovirus 3-16 Intertypic Recombinant Isolated in Argentina from an Infant Hospitalized with Acute Respiratory Infection. *J. Clin. Microbiol.* **2010**, *48*, 1494–1496, doi:10.1128/JCM.02289-09.

38. Arroyo-Cabrales J; Álvarez-Castañeda ST Corynorhinus rafinesquii. The IUCN Red List of Threatened Species 2017 2017.

39. Stadelmann, B.; Lin, L.-K.; Kunz, T. H.; Ruedi, M. Molecular phylogeny of New World Myotis (Chiroptera, Vespertilionidae) inferred from mitochondrial and nuclear DNA genes. *Mol. Phylogenet. Evol.* **2007**, *43*, 32–48, doi:10.1016/j.ympev.2006.06.019.

Table S1: Tentative viruses names, type of sample and localization of *Pipistrellus* group.

Tentative virus name	**Type of sample**		**Localization**
	OPS	SS	
Bat mastadenovirus *P.pygmaeus* 2 160725		X	Sevilla
Bat mastadenovirus *P.pygmaeus* 11 160725		X	Sevilla
Bat mastadenovirus *P.pygmaeus* 14 160725	X	X	Sevilla
Bat mastadenovirus *P.pygmaeus* 15 160725	X	X	Sevilla
Bat mastadenovirus *P.pygmaeus* 21 160725		X	Sevilla
Bat mastadenovirus *P.pygmaeus* 5 160811	X		Sevilla
Bat mastadenovirus *P.kuhlii* 1 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 3 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 6 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 16 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 17 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 20 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 22 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 40 160517	X		Córdoba
Bat mastadenovirus *P.kuhlii* 15 160518	X		Córdoba
Bat mastadenovirus *P.kuhlii* 2 160622	X		Huelva
Bat mastadenovirus *P.kuhlii* 11 160622		X	Huelva
Bat mastadenovirus *P.pygmaeus* 15 160622	X	X	Huelva
Bat mastadenovirus *P.kuhlii* 18 160622	X		Huelva
Bat mastadenovirus *P.pygmaeus* 19 160622		X	Huelva
Bat mastadenovirus *P.pygmaeus* 29 160622	X		Huelva
Bat mastadenovirus *P.pygmaeus* 31 160622		X	Huelva
Bat mastadenovirus *P.pygmaeus* 37 160622		X	Huelva
Bat mastadenovirus *P.pygmaeus* 19 160628		X	Huelva
Bat mastadenovirus *P.pygmaeus* 20 160628	X		Huelva
Bat mastadenovirus *P.pygmaeus* 23 160628		X	Huelva
Bat mastadenovirus *P.pygmaeus* 27 160628	X		Huelva
Bat mastadenovirus *P.pygmaeus* 6 070616		X	Lugo

Table S2: Tentative viruses names, type of sample and localization of *Nyctalus* group

Tentative virus name	Type of sample		Localization
	OPS	SS	
Bat mastadenovirus *N. lasiopterus* K01076	x		Cádiz
Bat mastadenovirus *N. lasiopterus* K01093	x		Sevilla
Bat mastadenovirus *N. lasiopterus* K01081	x		Cádiz
Bat mastadenovirus *N. lasiopterus* K01089	x		Cádiz
Bat mastadenovirus *N. lasiopterus* K00911		x	La Rioja
Bat mastadenovirus *N. lasiopterus* K01024	x		Cádiz
Bat mastadenovirus *N. lasiopterus* K01502		x	Málaga
Bat mastadenovirus *N. lasiopterus* K01504		x	Málaga
Bat mastadenovirus *N. lasiopterus* 4- 050701	x		Sevilla
Bat mastadenovirus *N. lasiopterus* 8- 050701	x		Sevilla
Bat mastadenovirus *N. lasiopterus* K01317	x		Sevilla
Bat mastadenovirus *N.leisleri* 00954		x	Málaga
Bat mastadenovirus *N. lasiopterus* K01508		x	Málaga
Bat mastadenovirus *N. lasiopterus* K01535		x	La Rioja
Bat mastadenovirus *N. lasiopterus* K01550	x		Málaga
Bat mastadenovirus *N.leisleri* 00964		x	La Rioja
Bat mastadenovirus *N. lasiopterus* K01530		x	La Rioja
Bat mastadenovirus *N.leisleri* 3-070704		x	Gerona
Bat mastadenovirus *N.leisleri* 1-080616	x		Navarra
Bat mastadenovirus *N. noctula* 13341	x		Navarra
Bat mastadenovirus *N. noctula* 13481	x		Navarra
Bat mastadenovirus *N. noctula* 13483	x		Navarra
Bat mastadenovirus *N. lasiopterus* K1849	x		Cádiz

Table S3: Tentative viruses names, type of sample and localization of *Hypsugo* and *Myotis* groups

Host	Tentative virus name	Type of sample		Localization
		OPS	SS	
Hypsugo group	Bat mastadenovirus H. savii 6 070704		X	Gerona
	Bat mastadenovirus H. savii 2 070613		X	Cáceres
	Bat mastadenovirus H. savii 2 070703		X	Gerona
Myotis group	Bat mastadenovirus M. emarginata 5 080703	X		Vizcaya
	Bat mastadenovirus M. emarginata 8 080703	X		Vizcaya
	Bat mastadenovirus M. emarginata 15 080703	X		Vizcaya
	Bat mastadenovirus M. bechsteinii 1 080619	X		Navarra
	Bat mastadenovirus M. myotis 6 080623	X		Córdoba

491 Table S4: Tentative viruses names, type of sample and localization of *Rhinolophus* group.

Tentative virus name	Type of sample		Localization
	OPS	SS	
Bat mastadenovirus R.ferrumequinum 1 40622	X		Valencia
Bat mastadenovirus R.ferrumequinum 1 070704		X	Gerona
Bat mastadenovirus R.euriale 12 080623	X		Córdoba
Bat mastadenovirus R.euriale 19 080623	X		Córdoba
Bat mastadenovirus R.euriale 5 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 19 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 23 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 25 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 5 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 23 080707	X		Vizcaya
Bat mastadenovirus R.euriale 25 080623	X		Córdoba
Bat mastadenovirus R.euriale 18 080623	X		Córdoba
Bat mastadenovirus R.ferrumequinum 30 080623	X		Córdoba
Bat mastadenovirus R.euriale 2 40409	X		Valencia
Bat mastadenovirus R.euriale A15	x		Valencia

492