BRIEF REPORT

Detection of alpha and betacoronaviruses in multiple Iberian bat species

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Abstract Bat coronaviruses (CoV) are putative precursors of the severe acute respiratory syndrome (SARS) CoV and other CoV that crossed the species barrier from zoonotic reservoirs into the human population. To determine the presence and distribution of CoV in Iberian bats, 576 individuals of 26 different bat species were captured in 13 locations in Spain. We report for the first time the presence of 14 coronaviruses in 9 Iberian bat species. Phylogenetic analysis of a conserved CoV genome region (RdRp gene) shows a wide diversity and distribution of alpha and betacoronavirus in Spain. Interestingly, although some of these viruses are related to other European BatCoV, or to Asian CoV, some of the viruses found in Spain cluster in new groups of α and β CoV.

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The emergence of infectious diseases is a major threat to global public health in this century (WHO, World Health Report http://www.who.int/whr/previous/en/index.html) and many of these new infectious human diseases are caused by viruses emerging from wildlife. In the last 50 years, more than 30 new infectious human diseases have been identified (WHO, World Health Report http://www.who.int/whr/previous/en/index.html) including the Severe Acute Respiratory Syndrome (SARS). The aetiological agent of this disease was identified as a previously unknown coronavirus (SARS-CoV) [1] and BatCoV are putative precursors of SARS-CoV [2]. The outbreak of SARS-CoV and subsequent identification of two additional human coronaviruses (HCoV-NL63[3] and HCoV-HKU1 [4]) has drawn human and animal health attention to *Coronavirinae* subfamily, that includes three genera, *Alphacoronavirus* (α CoV), *Betacoronavirus* (β CoV) and *Gammacoronavirus* (γ CoV), replacing the classical groups 1, 2 and 3 [5] (http://talk.ictvonline.org/media/g/vertebrate-2008/default.aspx).

The relevance and possible re-emergence of the pandemic SARS-CoV and other emerging viruses of zoonotic origin have activated surveillance systems of hazard agents in wild animals, including bats. As a result of these studies, bats have been described as putative reservoirs for some emerging viruses affecting humans [6]. BatCoV are putative precursors of CoV affecting humans and mammals [7, 8], including SARS-CoV [2] and other CoV that crossed the species barrier from zoonotic reservoirs into the human population [9]. In fact, association of some of these CoV to certain bat species has been suggested [10, 11], reinforcing the notion that there may be a relationship between some BatCoV and their hosts. Nowadays the presence of CoV has been shown in bats in China [12, 13], North and South America [14–17], Africa [18] and a number of regions in

Europe [11, 19–21] but not the Iberian Peninsula (Spain and Portugal), which is a bridge for European and African bat populations [22]. Thus, surveillance of wildlife reservoirs of putative zoonotic CoV is necessary, not only to unveil the ecology of these viruses, but also to permit early detection of viruses that might pose a threat to human health.

To determine the presence and distribution of putative zoonotic CoV in Iberian bats, 576 individuals from 26 bat species were captured and sampled in 13 different locations throughout Spain during 2004-2007 (Fig. 1). These samples were collected in the context of bat rhabdoviruses and lyssaviruses Surveillance Program in Spain. Most of the sampled bat species are also distributed across Europe, but *Eptesicus isabellinus* is a meridional serotine bat restricted to North Africa and the Iberian Peninsula [23], and *Myotis escalerai* is endemic in the Iberian Peninsula. Bats were caught with mist nets mainly as they left diurnal roost and by hand with polyethylene butterfly nets within roosts. Oro-pharyngeal swabs (n=390) between 2004 and 2007 as well as faecal samples from individual bats (n=216) in 2007 were taken before bats were released (Table 1).

Oro-pharyngeal swabs collected between 2004 and 2006 were preserved in 1 ml of lysis buffer (4 M GuSCN (Sigma), OS% N-lauroyl Sarcosine (Sigma), 1 mM dithiothreitol (DTT, Sigma), 25 mM Sodium Citrate and 20 pg/tube Glycogen (Boehringer Mannheim). Oro-pharyngeal swabs and faeces collected in 2007 were preserved in both 1 ml of lysis buffer and 1 ml of viral transport medium (VTM) (Eagle's minimal essential medium (EMEM) supplemented with 10 UI/ml of penicillin, 10 µg/ml of streptomycin, 160 µg/ml of gentamicin, 50 UI/ml of mycostatin and 1% of

bovine serum albumin). All samples were frozen at $-80°C$ before sending them to the Rabies Reference Laboratory at the Centro Nacional de Microbiología, ISCIII in Madrid. Faecal samples were clarified by centrifugation. Total nucleic acid was extracted from a 200 µl aliquot of each specimen for PCR assays and the rest were stored to $-80°C$ in two different aliquots. Final pellets were always resuspended to 55 µl of water.

A pan-coronavirus nested PCR was designed in the RdRp gene. A total of 5 µl of extracted RNA was added to 45 µl of reaction mixture of OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA) containing 200 µM dNTPs and 60 pmol of generic CoV-specific degenerated primers (forward 5′-CARATGAATYTIAARTAYGC-3′ and reverse 5′-TGYTGWGARCAAAAYTCRTG-3′) and following manufacturer indications. Amplifications were carried out into thin-walled reaction tubes (Sorenson, BioScience, UT) in a PTC-200 (Peltier Thermal Cycler, MJ Research, Watertown, MA). Nested PCR amplifications were performed using 2 µl of first amplification product and 48 µl of reaction mixture containing 60 mM Tris-HCl (pH 8.5), 15 mM $(NH4)_2SO_4$, 200 µM dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ), 3 mM $MgCl_2$, 35 pmol of generic CoV-specific degenerated primers (forward 5′-ATGGGWTGGGAYTAYCCIAARTG-3′ and reverse 5′-ACRTTRTTYTGRWARTA-3′) and 1.25 U AmpliTaq DNA Polymerase (Perkin-Elmer Cetus, Norwalk). Amplification product size of 512 nt was visualized by agarose gel electrophoresis and sequenced directly in both directions using an automated ABI PRISM 377 model sequencer. For phylogeny reconstruction, consensus sequences were aligned together with others obtained from public genomic databases using the program CLUSTAL X (version 1.83) (Table 2). A Bayesian phylogenetic inference was obtained using Mr Bayes version 3.1 [24] with random starting trees without constraints. For the analyses GTR substitution model, gamma estimation and two simultaneous runs of 10^7 generations were done, each with four Markov chains, and the trees were sampled every 100 generations. Amino acid identity was calculated with MEGA 4 using the pairwise deletion option. The alignment comprised the same 396 bp of the RdRp gene used for the phylogenetic reconstruction.

A total of 26 out of the 30 known bat species known for Iberian Peninsula were screened for CoV and 14 samples taken from 9 bat species, all included in the family Vespertilionidae, were positive for CoV RNA (Tables 1 and 2). Twelve of them were found within faecal samples (5.5%) of 7 different bat species in 6 locations and two were obtained in oral samples (0.5%) of 2 other different species in the same location (Table 1). In view of these results, it is not surprising that the presence of CoV RNA is significantly more frequent in faeces than in oral cavity

Fig. 1 Geographical location of bat capture sites in Spain. 1: A Coruña, 2: Lugo, 3: La Rioja, 4: Gerona, 5: Castellón, 6: Valencia, 7: Alicante, 8: Málaga, 9: Cádiz, 10: Sevilla, 11: Huelva, 12: Cáceres, 13: Menorca. The stars show the locations where positive samples were found

Table 1 Results of detection of CoV RNA in faecal or oral samples of bats collected in Spain

Bat Species	Faecal samples positive/no. tested	Oral samples positive/no. tested	Location	Genus
Barbastella barbastellus	0/4	0/2	3, 4	
Eptesicus isabellinus	1/8	NA	10[a]	β
Eptesicus serotinus	0/7	NA	1, 12	
Hypsugo savii	2/26	0/10	2, 4[a], 12[a,b]	α,β
Miniopterus schreibersii	0/2	1/71	2, 5, 6[a], 7, 8, 9, 12, 13	α
Myotis alcathoe	0/1	NA	3	
Myotis bechsteinii	0/2	0/3	4, 8	
Myotis blythii	NA	1/11	6[a], 7, 12	α
Myotis capaccinii	NA	0/14	5, 6, 13	
Myotis daubentonii	1/39	0/52	2, 3, 8[a], 11, 12	α
Myotis emarginatus	NA	0/2	8, 12	
Myotis escalerai	NA	0/15	11, 7	
Myotis myotis	1/1	0/17	6, 7, 8[a], 12	α
Myotis mystacinus	0/5	NA	2, 3	
Myotis nattereri	0/3	0/3	4	
Nyctalus lasiopterus	5/37	0/137	3, 8[a,b], 9, 10, 11	α
Nyctalus leisleri	0/23	0/11	2, 3, 4, 8	
Pipistrellus kuhlii	1/4	0/6	8, 12[a]	α
Pipistrellus pipistrellus	0/3	0/1	3, 12	
Pipistrellus pygmaeus	NA	0/1	12	
Pipistrellus sp.	1/29	0/5	1, 2, 4, 8, 12[a,b]	α
Plecotus auritus	0/7	NA	1, 3, 4	
Plecotus austriacus	0/7	0/10	4, 11, 12	
Rhinolophus euryale	NA	0/13	6, 7, 12	
Rhinolophus ferrumequinum	0/3	0/5	4, 8, 12	
Rhinolophus hipposideros	0/4	NA	4, 12	
Rhinolophus mehelyi	NA	0/1	12	
Total	12/216	2/390	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13	α,β

NA no samples available

[a] Locations where positives samples were found

[b] These samples were collected in different localities than other positive samples with the same number location

(p<0.0001, FISHER EXACT TEST). It is of interest that none of the viruses has been found in oropharingeal and faecal samples of the same individual, when both samples were available. This fact may indicate either that the infection was at different stage in the different individuals at the time of sampling or that replication of virus may take place independently in the intestinal and respiratory tracts [25]. Most of the CoV RNA sequences found in faecal samples (83%) correspond to α CoV, the remaining two belonging to β CoV. All CoV RNAs from oral samples (100%) were found to contain viral RNA sequences corresponding to α CoV. In agreement with all previous studies [7], none of the coronavirus detected in Spanish bats belong to group γ.

The phylogenetic analysis of Spanish BatCoV was performed using 396nt out of the 512 nt RT-PCR amplified fragments. 116nt fragment information was lost to allowed us include more sequences from other European countries and other continents deposited in GenBanK to perform a meaningful analysis. This small part of the RdRp gene has been previously used, and sufficiently represents the full gene information, for phylogenetic analysis of BatCoV [11, 14, 20]

The phylogenetic reconstruction showed 6 different lineages of Spanish BatCoV (Fig. 2). BatCoV A and B were closely related to other α BatCoV found in China [12], although they appeared to display certain genetic differentiation (Fig. 2). *Myotis daubentonii*-associated CoV H, and *Pipistrellus*-associated CoV K, clustered respectively with lineages 4 and 3 of α CoV previously described in Germany and are hosted by the same bat species or genera [11] (Fig. 2). BatCoV L was closely

Table 2 Data of interest related to the 91 coronavirus sequences used for the generation of the phylogenetic tree

Access no	Host species	Country	Genus	Cluster
DQ249221	Bat	China	β	HKU5
DQ249219	Bat	China	β	HKU5
DQ249218	*Pipistrellus* sp.	China	β	HKU5
DQ648809	Bat	China	β	
DQ648807	Bat	China	β	
DQ249217	*Pipistrellus* sp.	China	β	HKU5
DQ648819	Bat	China	β	
DQ249215	*Tylonycteris*	China	β	HKU4
DQ249214	*Tylonycteris*	China	β	HKU4
DQ074652	*Tylonycteris*	China	β	HKU4
DQ249216	*Tylonycteris*	China	β	HKU4
DQ648803	Bat	China	β	
HQ184059	*Hypsugo savii*	Spain	β	
HQ184062	*Eptesicus isabellinus*	Spain	β	
GQ404795	*Rhinolophus hipposideros*	Slovenia	β	
GQ404796	*Rhinolophus hipposideros*	Slovenia	β	
GQ404797	*Rhinolophus hipposideros*	Slovenia	β	
DQ022305	*Rhinolophus sinicus*	China	β	
NC_009696	*Rhinolophus macrotis*		β	
NC_004718	Human		β	
NC_009021	*Rousettus leschenaulti*	China	β	HKU9
NC_006577	Human		β	
NC_006852	Mouse		β	
NC_007732	Pig		β	
NC_005147	Human		β	
EF544563	*Myotis occultus*	USA	α	
EF544565	*Myotis occultus*	USA	α	
HQ184049	*Miniopterus schreibersii*	Spain	α	
HQ184050	*Myotis blythii*	Spain	α	
DQ648838	Bat	China	α	
DQ648855	*Rhinolophus ferrumequinum*	China	α	
DQ648854	*Rhinolophus ferrumequinum*	China	α	
NC_003436	Pig		α	
EU375862	*Myotis dasycneme*	Germany	α	
EU375859	*Myotis dasycneme*	Germany	α	
EU375858	*Myotis dasycneme*	Germany	α	
EU375855	*Myotis dasycneme*	Germany	α	
EU375863	*Myotis dasycneme*	Germany	α	
EU375861	*Myotis dasycneme*	Germany	α	
EU375856	*Myotis dasycneme*	Germany	α	
EU375854	*Myotis dasycneme*	Germany	α	
EU375857	*Myotis dasycneme*	Germany	α	
EU375865	*Myotis bechsteinii*	Germany	α	
EU375853	*Myotis bechsteinii*	Germany	α	
EU375860	*Myotis bechsteinii*	Germany	α	
EU375869	*Pipistrellus nathusii*	Germany	α	
EU375864	*Pipistrellus nathusii*	Germany	α	
EU375870	*Pipistrellus pygmaeus*	Germany	α	

Table 2 continued

| Access no	Host species	Country	Genus	Cluster
EU375868	*Pipistrellus pygmaeus*	Germany	α	
EU375867	*Pipistrellus pygmaeus*	Germany	α	
HQ184060	*Pipistrellus* sp.	Spain	α	
DQ648822	Bat	China	α	
DQ648821	Bat	China	α	
DQ648824	Bat	China	α	
DQ648823	Bat	China	α	
EU375875	*Myotis daubentonii*	Germany	α	
EU375873	*Myotis daubentonii*	Germany	α	
EU375874	*Myotis daubentonii*	Germany	α	
EU375872	*Myotis daubentonii*	Germany	α	
EU375866	*Myotis daubentonii*	Germany	α	
HQ184056	*Myotis daubentonii*	Spain	α	
EU375871	*Myotis daubentonii*	Germany	α	
DQ648833	*Myotis ricketti*	China	α	
DQ249224	*Myotis ricketti*	China	α	HKU6
DQ648837	*Myotis ricketti*	China	α	
DQ249235	*Rhinolophus sinicus*	China	α	HKU2
DQ249213	*Rhinolophus sinicus*	China	α	HKU2
DQ648840	Bat	China	α	
DQ249228	*Miniopterus* sp.	China	α	HKU8
EU834954	*Miniopterus australis*	Australia	α	
EU834952	*Miniopterus australis*	Australia	α	
EU834955	*Miniopterus schreibersii*	Australia	α	
EU834953	*Rhinolophus megaphyllus*	Australia	α	
DQ648835	*Miniopterus schreibersii*	China	α	
DQ648796	Bat	China	α	
DQ648797	Bat	China	α	
DQ249226	*Miniopterus magnater*	China	α	HKU7
HQ184061	*Hypsugo savii*	Spain	α	
HQ184051	*Nyctalus lasiopterus*	Spain	α	
HQ184054	*Nyctalus lasiopterus*	Spain	α	
HQ184053	*Nyctalus lasiopterus*	Spain	α	
HQ184052	*Nyctalus lasiopterus*	Spain	α	
HQ184055	*Nyctalus lasiopterus*	Spain	α	
HQ184057	*Myotis myotis*	Spain	α	
HQ184058	*Pipistrellus kuhlii*	Spain	α	
EU834951	*Myotis macropus*	Australia	α	
NC_002645	Human		α	
NC_005831	Human		α	
AY994055	Cat		α	
NC_002306	Pig		α	
NC_001451	Chicken		γ	
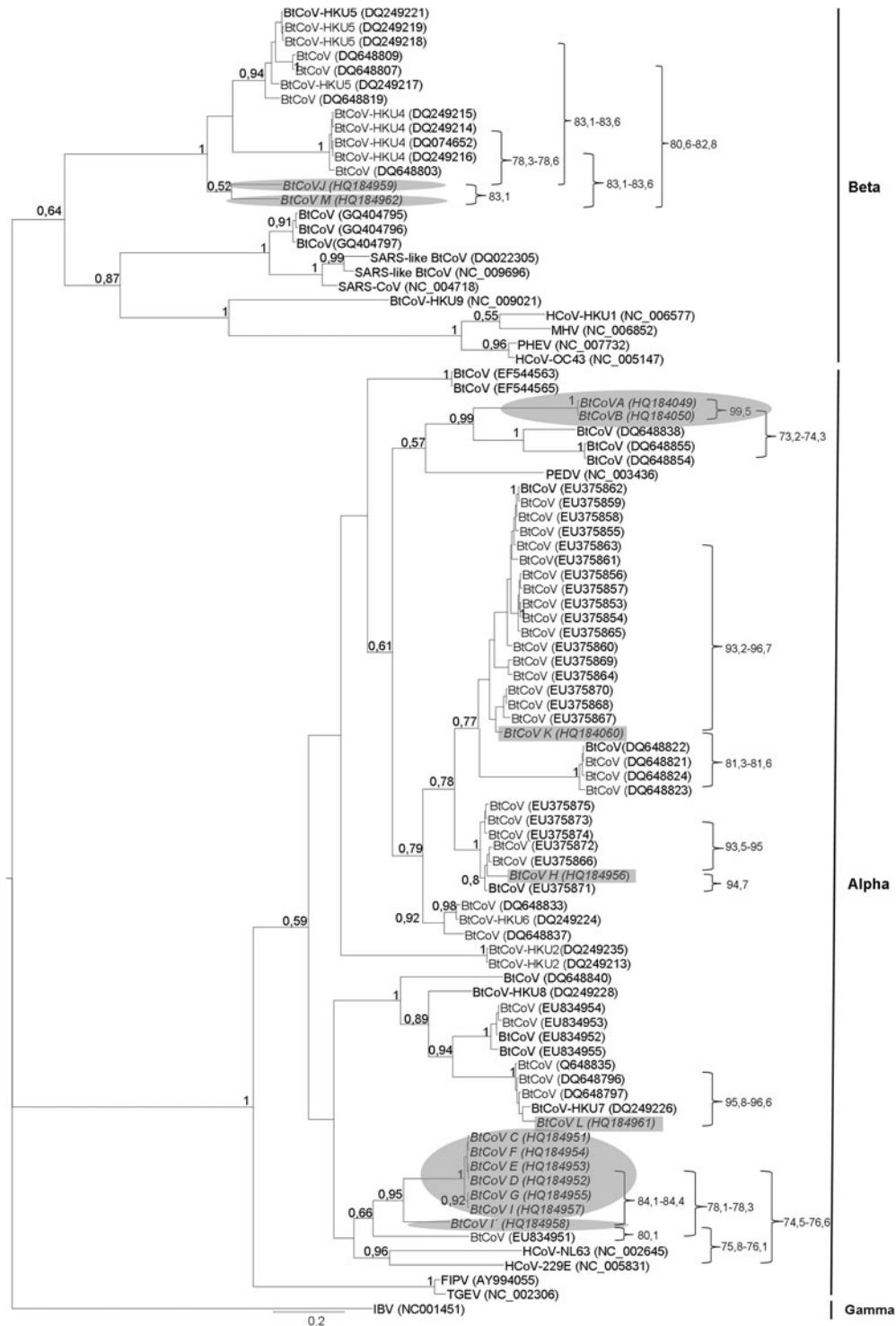
Fig. 2 CoV phylogenetic reconstruction based on 396 bp of the RdRp gene including 14 Spanish CoV from different bat species and 77 alpha, beta and gammacoronaviruses obtained from GenBank. Accession numbers are shown in brackets. BatCoV detected in Spain are highlighted in italics. For the analyses GTR substitution model, gamma estimation and two simultaneous runs of 10^7 generations were done, each with four Markov chains, and the trees were sampled every 100 generations. First 25% trees were excluded as burn-in from the analysis. Significant posterior probabilities are indicated. Complementary information about sequences used in this phylogenetic reconstruction are shown in Table 2. Positive samples described in this work are shown in shaded rectangles and ovals. The new alpha and betacoronavirus groups described in this work are shown in shaded ovals. Amino acid identity was calculated with MEGA 4 using the pairwise deletion option. The alignment comprised the same 396 bp of the RdRp gene used for the phylogenetic reconstruction. Amino acid identities across 132 amino acids are indicated next to the brackets that links every Spanish BatCoV to the phylogenetic neighbours

related to cluster HKU7 described in China [10]. BatCoV C, D, E, F, G and I belonged to the same lineage and showed an intriguing new independent cluster (significant posterior probability = 0.95) including BatCoV I' (Fig. 2). In addition, BatCoV J and M were genetically related to β CoV, although they did not really associate with any of their previously described linages. Sequences corresponding to two different groups of CoV were found in the same bat species (*Hypsugo savii*) as it was also found for other bat species elsewhere [12]. Spanish BatCoV described here were also classified calculating amino acid distances of these viruses from phylogenetic neighbours and related reference species [21]. Amino acid distance criteria recently described for separating RdRp grouping units (RGU) were adopted [21]. Interestingly, the amino acid distance criteria (>4.8% for alphacoronavirus and >6.3% for betacoronavirus) reinforced the presence of the new group of alphacoronaviruses mentioned above including Spanish BatCoV C, D, E, F, G and I; and additionally showed four new tentative groups (Fig. 2). BatCoV A and B were included in a new alphacoronavirus group and BatCoV I' constituted another independent alphacoronavirus group. BatCoV J, and M represented two new betacoronavirus groups and BatCoV H, K and L remained as part of several established species (Fig. 2).

It has been previously suggested that some CoV associate to certain bat species [10, 11]. However, we found that different bat species from the same colony or location harbour CoV of the same genetic lineage (BatCoV A and B; G and I), indicating a greater diversity and higher complexity than previously described for the ecology of BatCoV. Similar exceptions were found in China and South America [12, 16, 26] and could also be observed with Australian BatCoV (Fig. 2 and Table 2).

In conclusion, previous studies showed the presence of BatCoV in Europe. However, to our knowledge, this is the first report describing the presence of CoV RNA in Iberian bat species. Phylogenetic data indicate high diversity, wide distribution and complex ecology of CoV in bats captured in diverse Spanish locations. The sequences reported herein provide new insights into the diversity of coronaviruses and describe new phylogenetic linages that appear to diverge from all genotypes previously detected in other European locations. Future studies should clarify whether such apparently high diversity reflects the bio-geographical peculiarities of the Iberian Peninsula or not. This study contributes with a new dataset to the global surveillance of emerging BatCoV with pathogenic potential in humans. Our data reinforce the notion that the ecology and transmission of CoV in bat reservoirs is far from being completely understood and that more studies will be necessary to evaluate the magnitude of the potential threat that these viruses pose to human health.

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