The SARS-like coronaviruses: the role of bats and evolutionary relationships with SARS coronavirus

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——— SUMMARY ———

Bats represent an order of great evolutionary success, with elevated geographical diffusion and species diversity. This order harbors viruses of high variability which have a great possibility of acquiring the capacity of infecting other animals, including humans. Bats are the natural reservoir for several viruses genetically closely related to the SARS-coronavirus which is the etiological agent of severe acute respiratory syndrome (SARS), a human epidemic which emerged in China in 2002-2003. In the last few years, it has been discovered that the association between coronaviruses and bats is a worldwide phenomenon, and it has been hypothesised that all mammalian coronaviruses were derived from ancestral viruses residing in bats. This review analyzes the role of bats as a reservoir of zoonotic viruses focusing more extensively on SARS-related coronaviruses and taking into account the role of African and European strains in the evolutionary history of these viruses.

KEY WORDS: Bat, SARS, Coronavirus

INTRODUCTION

In mammalians, Chiroptera is one of the most important orders involved in the emergence and diffusion of viruses in animals, and between animals and humans. Several characteristics of the bat species, such as the ability to fly, the coexistence of many individuals in large colonies, a diet composed of all types of foods, the ability to cover long distances during migration as well as many others, have allowed bats to be found all over the world (except in Antarctica and a few oceanic islands), occupying the most varied ecological niches. This great evolutionary success underlies the remarkable propensity of these animals to favor, as hosts and reservoirs, the diffusion of a wide variety of viruses.

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In past years, several viruses infecting humans have been linked to bats: Rhabdoviridae, Orthomyxoviridae, Coronaviridae, Flaviviridae, Filoviridae and others (Calisher *et al.*, 2006). With some exceptions, the direct transmission of viruses from bats to humans is uncommon. Instead, bats more frequently act like reservoirs of ancestral viruses which, through a species jump, arrive in secondary hosts where they acquire a tropism for the human host.

Due to the elevated geographical diffusion and the elevated species diversity of bats, this order harbors viruses of high variability which have a greater possibility of acquiring the capacity of infecting other animals, including humans.

In recent years, several anthropogenic and natural changes in the environment, due to deforestation, alteration of natural habitats, changes in animal diversity and climatic events, have shifted the ecology of bats and have exposed humans to new pathogens (Wang *et al.*, 2006). In fact, the last twenty years have seen an increase in the number of outbreaks caused by pathogens with bats as the reservoir of infection (Calisher *et al.*, 2006).

Of the human viral epidemics which have emerged in the last ten years, one of the most important is the outbreak of severe acute respiratory syndrome (SARS) which emerged in China in 2002-2003, rapidly causing a global epidemic involving humans. In two consecutive events (the main SARS epidemic of 2002-2003 and a series of sporadic infections occurring in the winter of 2003-2004), SARS caused more than 8000 infections and 700 deaths (Centers for Disease Control and Prevention (CDC), 2003; Peiris *et al.*, 2004; Skowronski *et al.*, 2005).

Several studies have shown that a new coronavirus is the etiological agent of SARS, the SARS-CoV (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Rota *et al.*, 2003). Successively, it has also been shown that bats might be the natural reservoir for several viruses closely related genetically to the SARS-CoV, known as SARS-like coronaviruses (SARS-like CoVs), and that all mammalian coronaviruses were derived from ancestral viruses residing in bats (Shi *et al.*, 2008; Woo *et al.*, 2009).

Furthermore, the association between coronaviruses and bats is a worldwide phenomenon: after the initial discovery of this virus in Chinese bats (Lau *et al.*, 2005; Li *et al.*, 2005), subsequent studies confirmed its presence in North and South America, Europe and Africa (Dominguez *et al.*, 2007; Brandao *et al.*, 2008; Carrington *et al.*, 2008; Gloza-Raush *et al.*, 2008; Misra *et al.*, 2009; Pfefferle *et al.*, 2009; Tong *et al.*, 2009; Donaldson *et al.*, 2010; Drexler *et al.*, 2010; Quan *et al.*, 2010; Reusken *et al.*, 2010; Rihtaric *et al.*, 2010; Watanabe *et al.*, 2010; Balboni *et al.*, 2011).

This review will analyze the role of bats as reservoirs of zoonotic viruses focusing more extensively on SARS-related coronaviruses: the discovery of the coronavirus in bats, the genomic features of the several SARS-like CoVs, and the gradual and ongoing identification of new viral strains in bats in different parts of the world.

In particular, the role of SARS-like CoVs residing in African and European bats will be taken into account in the evolutionary history of these viruses, in the light of the typical genome divergences that these viruses have when compared to Asian SARS-like CoVs.

Bats and their role in viral spread

Bats are one of the longest living mammalian orders, and probably constitute the order with the most diversity between species (Altringham, 1996a). They originated early (50 to 52 million years ago) and the different species have changed relatively little over time compared to mammals of other taxa (Calisher *et al.*, 2006).

Bats constitute the second largest order of mam-

TABLE 1 - *Bat classification.*

Order	Suborder	Family	Common name
Chiroptera	Megachiroptera	Pteropodidae	Flying foxes and other Old World fruit bats
	Microchiroptera	Vespertilionidae	Vesper bats or evening bats or common bats
		Phyllostomidae	American leaf-nosed bats, including vampire bats
		Rhinolophidae	Horseshoe bats
		Hipposideridae	Old World leaf-nosed bats
		Molossidae	Free-tailed bats
		Emballonuridae	Sheath-tailed bats or sac-winged bats
		Nycteridae	Slit-faced bats or hollow-faced bats
		Mormoopidae	Leaf-chinned bats
		Megadermatidae	False vampire bats
		Natalidae	Funnel-eared bats
		Rhinopomatidae	Mouse-tailed bats
		Thyropteridae	Disk-winged bats
		Mystacinidae	New Zealand short-tailed bats
		Furipteridae	Smoky bats
		Noctilionidae	Bulldog bats or fisherman bats
		Craseonycteridae	Hog-nosed or bumblebee bat
		Myzopodidae	Old World sucker-footed bat

mals and, with approximately 1000 species, make up 20% of the species in the Class *Mammalia.* Bats are classified in the order *Chiroptera,* which is subdivided in two suborders, *Mega-* and *Microchiroptera*, commonly referred to as megabats and microbats.

The suborder *Megachiroptera* contains only one family (Pteropodidae or flying foxes), which includes only plant-eating large bats (20-1500 g), confined to Africa, tropical Asia and Indo-Australasia. Instead, the Suborder *Microchiroptera* contains 17 families which include the majority of small bats (1.5-150 g). They are found on every continent except Antarctica and a few oceanic islands, and are omnivorous (Table 1) (Altringham, 1996a; Calisher *et al*., 2006; Wang *et al*., 2006).

As regards the geographical distribution of bats, a decrease in species richness is seen with an increase in latitude. Therefore, there is greater species richness in proximity to the equator due to greater major primary biological productivity as there is an increase in light intensity, temperature and the growing season (Altringham, 1996d).

Owing to the diversity of bat species and their unique biological and ecological characteristics (all bats fly, some are abundant and widely distributed, many are highly gregarious, they are predators and are preyed upon at the same time), they can become the hosts for a large number of infectious agents having medical importance (Calisher *et al*., 2006; Wang *et al*., 2006; Donaldson *et al*., 2010;).

The transmission of viruses from bats to humans can occur in a variety of ways: direct contact (bites and scratches), inhalation of infectious particles by humans (secretions and guano), arthropod vectors, consumption of bat meat in some parts of the world (China, Guam and some parts of Asia) and secondary vertebrate hosts which serve as amplifying hosts (Wang *et al*., 2006). Therefore, bats represent an important reservoir for many zoonotic viruses which usually cause persistent infections in bats themselves. Furthermore, with some exceptions, the transmission of viruses from bats to humans requires a second intermediate animal host, and direct transfer of the pathogen from bats to humans happens only rarely (Calisher *et al*., 2006).

As mentioned above, the capability of bats to host several viral species and transmit viruses to other animals, including humans, is favored by various biological and ecological characteristics; the main ones are:

a) The high population densities and the habit of roosting provides the opportunity for disease-causing pathogens to invade and spread rapidly, with the possibility of intra- and interspecies transmission of infectious agents amongst themselves through direct contact, or aerosol or arthropod vectors (Kunz *et al*., 2003c; Calisher *et al*., 2006; Wang *et al*., 2006; Woo *et al*., 2006).

The number of bats in each colony varies greatly from less than 10 to over 200,000 individuals (Wang *et al*., 2006), and many bat species may share the same habitats. Bats exhibit a wide range of fidelity to their roosts. This variation is often reflected in the type of roost, stage of life and form of social organisation. Many species of cavity- and foliage-roosting bats show low fidelity to specific roost size as compared to cave- and building-roosting bats, but they often all exhibit high levels of fidelity to roost areas (Kunz *et al*., 2003a). The roosting environment ranges from natural structures (caves, rock crevices, nests of birds, ants and termites, hollowed spaces in trees, or exposed on tree branches and trunks) to man-made structures (mines, tombs, buildings and bridges), and they can be temporary or permanent (Kunz *et al*., 2003a; Wang *et al*., 2006). The occupation of man-made habitats could bring the bats into closer association with humans and their companion animals or livestock with the possible transmission of some pathogens from peridomestic bats to humans and livestock (Wang *et al*., 2006).

b) The dietary habits of bats can be broadly divided into insectivorous, frugivorous, carnivorous, omnivorous and sanguivorous (Wang *et al*., 2006). Predatory bats could potentially acquire infectious agents from other animal species, such as birds and insects (Wang *et al*., 2006) but, on the other hand, bats are also preyed upon and they can transmit infectious agents to their predators.

c) The ability to fly has given bats the opportunity of going almost anywhere, freer from obstacles than land-based mammals (Woo *et al*., 2006). Some genera migrate between two sites to avoid unfavourable climatic conditions and/or to seek more favourable conditions for growth, covering

distances ranging from 200-300 km to almost 2000 km (Calisher *et al.*, 2006; Wang *et al.*, 2006). There are two types of migration: 1) long seasonal migration generally in a north-south direction and 2) short seasonal migration not necessarily in a north-south direction. Migration is particularly common in tree-roosting bats since tree holes are poor hibernation sites in the colder climates. Furthermore, female migration is more common than male (Altringham, 1996c; Kunz *et al.*, 2003b).

The evolution of migratory behaviour varies with latitude; in fact, migratory behaviour is more intense at higher latitudes than at lower latitudes, and a higher proportion of temperate bats exhibit migratory behaviour than do tropical species (Kunz *et al.*, 2003b).

Therefore, bats have a greater capacity to disperse quickly to new areas, such as islands, than do non-volant mammals, with the exception of humans. The migratory habits of some bats provide an opportunity for pathogens to cover long distances and bridge gaps between various species which might otherwise not be in contact (Kunz *et al.*, 2003c).

d) *Vespertilionidae* and *Rhinolophidae* bats have the ability of entering into daily torpor and seasonal hibernation to conserve energy during cool nights and winter months (Calisher *et al.*, 2006). The term torpor means a state in which an animal allows its body temperature to fall below its active homeothermic level. The fall in body temperature is slow and controlled, and permits conservation of energy. Hibernation is an extended form of torpor which occurs on a seasonal basis, lasting for days, weeks, or months, in response to a prolonged fall in ambient temperature or a reduction in the food supply (Altringham, 1996b). The capability of bats to enter into seasonal hibernation may also facilitate the diffusion of pathogens since it can allow the viruses to overwinter within the bats, thus facilitating their persistence in the environment (Calisher *et al.*, 2006).

Coronavirus

Coronaviruses (CoVs), order *Nidovirales* and family *Coronaviridae*, are a group of enveloped viruses with a linear, non-segmented, positive-sense, single-stranded RNA genome, having a characteristic crown morphology (Fauquet *et al.*, 2005). Until a few years ago, all viruses belonging to the family *Coronaviridae* were subdivided into only two genera: *Coronavirus* and *Torovirus*, and all viruses included in the genus *Coronavirus* were subdivided into only three antigenic groups. The recent discovery of a large variety of new coronaviruses in several host species has led the Coronavirus Study Group of the International Committee for Taxonomy of Viruses (ICTV) to propose a reclassification of the family *Coronaviridae* into two subfamilies: *Coronavirinae* and *Torovirinae*. In particular, the subfamily Coronavirinae now includes three genera: *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus*, which have replaced the traditional antigenic groups 1, 2, and 3, respectively. All the mammalian CoVs, including all bat coronaviruses detected to date and SARS-CoV, belong to the first and the second genera, *Alphacoronavirus* and *Betacoronavirus*, whereas all avian CoVs belong to the genus *Gammacoronavirus*. Within the *Gammacoronavirus* genus, there is only one exception represented by the Beluga whale coronavirus SW1 strain which has been identified in this aquatic mammal (Fauquet *et al.*, 2005; Woo *et al.*, 2009; http://www.ncbi.nlm.nih.gov/taxonomy). The viral genome is the largest known non-segmented viral RNA genome (approximately 30 kbp) and is subdivided into a variable number of open reading frames (6 to 9) which encode an ensemble of non-structural proteins with RNA-polymerase function (Pol-1a,b) and four principal structural proteins: Spike (S), Membrane (M), Envelope (E) and Nucleocapsid (N) proteins. In addition to these, some non-structural proteins, which are diverse in number, have uncertain functions and have a group-specific genome position, have been added (Fauquet *et al.*, 2005).

Briefly, the Pol-1a,b proteins are encoded from an open reading frame (ORF) which occupies 2/3 of the genome and presents highly conserved regions. The Spike glycoprotein, exposed on the virion surface, is responsible for recognising and binding with the cellular receptor, inducing neutralising antibody response and is encoded from an ORF with a highly variable sequence. The nucleocapsid protein is linked with genomic RNA and induces a cell-mediated immune response (Fauquet *et al.*, 2005).

Coronaviruses have diverse animal hosts ranging from mammalian (including humans) to avian species, and have a high frequency of recombi-

nation which, together with high mutation rates, may allow them to adapt to new hosts and ecological niches. The main targets are epithelial cells, causing mainly enteric and respiratory diseases of varying severity. Transmission occurs through fecal-oral secretions and excretions.

Sars and bat coronaviruses

The severe acute respiratory syndrome-associated coronavirus (SARS-CoV or hu-SARS-CoV) is the etiologic agent of the SARS disease (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003; Rota *et al.*, 2003) which caused a global epidemic in humans in 2002-2003, resulting in more than 8000 infections and 700 deaths (Centers for Disease Control and Prevention (CDC), 2003; Peiris *et al.*, 2004; Skowronski *et al.*, 2005).

From the initial investigations conducted in China after the outbreak of SARS, it seemed clear that marketplace animals may have been the source of the virus found in humans (Li *et al.*, 2006; Shi *et al.*, 2008). Evidence of infection was found in Himalayan palm civets (*Paguma larvata*), Chinese ferret badgers (*Melogale moschata*) and raccoon dogs (*Nyctereutes procyonoides*) which were found to carry the SARS-CoV.

In particular, it was seen that the viruses collected from masked palm civets in 2003 were different from those of 2004 indicating two separate animal-to-human transmissions: the first in the main SARS outbreak of 2002-2003 and the second in a series of sporadic infections in the winter of 2003-2004 (Song *et al.*, 2005; Shi *et al.*, 2008; Graham *et al.*, 2010).

However, subsequent epidemiological studies have shown that Palm civets and other marketplace animals may not be the primary reservoir hosts of SARS-CoV in nature, but merely a secondary amplifying host which increases the viral burden and provides ample contact with humans, thereby facilitating animal-to-human and human-to-human transmission. This hypothesis is supported by the fact that:

1) SARS-CoV was present only in market or farmed animals but not in those from the wild;
2) SARS-CoV genomes evolved rapidly in marketplace animals, suggesting that the virus was still adapting to these animals rather than persisting in equilibrium, as would be expected in a reservoir species (Wang *et al.*, 2006; Graham *et al.*, 2010).

Nonetheless, the finding of SARS-CoV in marketplace animals in China has provided evidence for interspecies transmission in the genesis of the SARS epidemic (Lau *et al.*, 2005).

The research of the wild animal reservoir of SARS-CoV subsequently led to the discovery of a great variety of coronaviruses belonging to the first two coronavirus genera in several bat species, first in Asia (Lau *et al.*, 2005; Li *et al.*, 2005; Poon *et al.*, 2005; Chu *et al.*, 2006; Tang *et al.*, 2006; Woo *et al.*, 2006; Woo *et al.*, 2007) and then in all the continents in which bats are found (Dominguez *et al.*, 2007; Brandao *et al.*, 2008; Carrington *et al.*, 2008; Gloza-Raush *et al.*, 2008; Misra *et al.*, 2009; Pfefferle *et al.*, 2009; Tong *et al.*, 2009; Donaldson *et al.*, 2010; Drexler *et al.*, 2010; Quan *et al.*, 2010; Reusken *et al.*, 2010; Rihtaric *et al.*, 2010; Watanabe *et al.*, 2010; Balboni *et al.*, 2011), whereas none of the bat coronaviruses discovered belonged to the third coronavirus genus.

These discoveries, in association with the ancestral nature of bat coronaviruses compared to coronaviruses from other animal species, have led us to assume that all mammalian coronaviruses belonging to *Alphacoronavirus* and *Betacoronavirus* were derived from ancestral viruses residing in bats (Vijaykrishna *et al.*, 2007; Woo *et al.*, 2009).

Currently, there is no evidence of SARS-CoV transmission from bats to humans (Stockman *et al.*, 2008), and the exact natural reservoir host for the progenitor virus of SARS-CoV may not have been determined as yet. However, a group of CoVs which are genetically closely related to the SARS-CoV have been identified in various species of horseshoe bats (*Rhinlophus* spp.) and in some other bat genera (Lau *et al.*, 2005; Li *et al.*, 2005; Tang *et al.*, 2006; Woo *et al.*, 2006; Lau *et al.*, 2007; Woo *et al.*, 2007; Pfefferle *et al.*, 2009; Tong *et al.*, 2009; Drexler *et al.*, 2010; Lau *et al.*, 2010; Quan *et al.*, 2010; Rihtaric *et al.*, 2010; Yuan *et al.*, 2010; Balboni *et al.*, 2011). These viruses were called SARS-like CoVs. These SARS-like CoVs together with human and marketplace animal SARS-CoV strains are included in the *Betacoronavirus* genus, in the severe acute respiratory syndrome-related coronavirus (SARS-related CoV) species (http://www.ictvonline.org/).

There are at least three hypotheses which explain the origin of the SARS-CoV:

1) some unknown intermediate hosts were responsible for the adaptation and transmission of the SARS-CoV from bats to marketplace animals or humans;

2) there is a SARS-like CoV closely related to the outbreak SARS-CoV strains in a non-bat animal host which is capable of direct transmission from a reservoir host to humans or marketplace animals;

3) a bat yet to be identified could be the direct reservoir of the human or marketplace animal SARS-CoV because it may have an efficient viral receptor (ACE2, see below) (Hou *et al.*, 2010).

With regard to the presence of coronaviruses in bats, there may be a coevolutionary relationship between some bat-CoVs and their hosts. Therefore, with very few exceptions, most bat coronaviruses seem to be species-specific, although one bat genus/species may more than one type of coronavirus (Wang *et al.*, 2006; Woo *et al.*, 2006; Cui *et al.*, 2007).

Bat-CoV seemed to be more closely associated with bat species than with sampling location; indeed, the same bat species living in different geographical locations can contain the same type of coronavirus and, at the same time, different species roosting in the same cave may carry different coronaviruses (Tang *et al.*, 2006; Gloza-Raush *et al.*, 2008; Reusken *et al.*, 2010). Therefore, virus-host associations could be used to predict geographic distributions of reservoir-borne CoVs (Drexler *et al.*, 2010).

Moreover, there seems to be a relationship between cornavirus infection and the age of the bats; in fact, it has been shown that young bats provide a susceptible population for amplifying the coronavirus and transmitting it to lactating females in maternity colonies whereas the virus would replicate less efficiently in adults than in young animals (Gloza-Raush *et al.*, 2008; Rihtaric *et al.*, 2010).

To date, SARS-like coronaviruses have been detected in China, Africa and Europe. With the exception of the African strains, all bat-SARS-like CoVs have been detected in some species of horseshoe bats (*Rhinolophus* spp.) where they cause acute, self-limiting infection of the gastrointestinal tract (Lau *et al.*, 2010). The detection of diverse SARS-like CoV strains in the different *Rhinolophus* species have suggested that

this novel group of coronaviruses is rapidly evolving and may easily cross the species barrier (Lau *et al.*, 2010).

Inside this bat genus, the Chinese horseshoe bat (*Rhinolophus sinicus*) probably plays an important role in the SARS-like CoV epidemiology. First, the highest virus prevalence detected until now was in *R. sinicus* (Shi *et al.*, 2008) and, in addition, two SARS-like-CoVs, Rs672 and Rp3, identified in this bat species (Li *et al.*, 2005; Yuan *et al.*, 2010) tightly clustered together and showed a potential recombinant breakpoint estimated at the nucleotide immediately after the start codon of the S gene, with the major parental regions phylogenetically closer to human-SARS-CoVs than to bat-SARS-like-CoVs and the minor parental region phylogenetically closer to bat-SARS-like-CoVs than to human-SARS-CoVs (Yuan *et al.*, 2010). It is possible that the recombination insertion of variant RBDs (Receptor-Binding Domains) may have mediated the initial cross-species transmission event from bats into other mammals (Graham *et al.*, 2010).

SARS-like-CoV genome

All bat-SARS-like CoV full genomes have a gene organization similar to human and civet SARS-CoVs; the genome is composed of a 5' non-coding leader sequence, polymerase complex ORF1ab, S, ORF3 (formed to ORF3a,b,c in Rf1 strain), E, M, ORF6, ORF7a,b, ORF8, ORF9a(N),b,c and 3' terminal non-coding region with a stem loop II-like motif (s2m) and a polyA tail (Figure 1a). Another genome characteristic shared by SARS and SARS-like coronaviruses is the presence of a conserved nucleotide sequence (5'-ACGAAC-3') functioning as a transcription regulatory sequence (TRS) located at the 3' end of the putative 5' leader sequence of each predicted gene start site (Marra *et al.*, 2003; Rota *et al.*, 2003). This motif is indispensable for the production of the subgenomic mRNAs and is identical in all SARS-CoV ORFs and in most bat-SARS-like CoV ORFs (Li *et al.*, 2005; Woo *et al.*, 2007).

Nevertheless, there are considerable diversities within the genome organisation of SARS-like CoVs and between these and human and civet SARS-CoVs (Fig.1b) (Shi *et al.*, 2008; Quan *et al.*, 2010). Major differences have been observed in nsp3 (third non-structural protein codified by ORF 1a,b), S, ORF3 and ORF8 regions which

showed the lowest nucleotide and amino acidic identities in comparison to the rest of the genome (Lau *et al.*, 2005; Shi *et al.*, 2008; Lau *et al.*, 2010). The principal genome differences between SARS and SARS-like viruses are located in ORF8 which is a frequent site for deletion. All Bat-SARS-like CoVs (except the HKU3-8 strains, GQ153543), civet-SARS-CoVs (except one strain) and early-phase SARS-CoVs harbor a 29-nts sequence which fuses ORF8a and ORF8b into a single ORF8. Instead, in successive-phase SARS-CoVs, this 29-nts does not exist and ORF 8 is subdivided into two distinct ORF8s (a and b), disrupting the functional expression of this open reading frame (Lau *et al.*, 2005; Oostra *et al.*, 2007; Shi *et al.*, 2008; Graham *et al.*, 2010; Lau *et al.*, 2010). The bat-SARS-like CoV strain HKU3-8 is an exception, having a short deletion of 26 bp which subdivides this ORF into three smaller ORFs (Lau *et al.*, 2010).

The S gene of bat-SARS-like CoVs presents a low percentage of sequence identity compared to human and civet SARS-CoVs, especially localized in the putative S1 domain. In the bat-SARS-like CoVs S1 domain, there are several insertions and deletions, mainly in the RBD region, which is critical for host-receptor binding and for immunity response (Lau *et al.*, 2005; Li *et al.*, 2005).

The nsp3 region of ORF1a is relatively variable in the genome of bat-SARS-like CoVs and, in particular, a deletion of 579nt in the nsp3 of the Rs672 strain has been identified, only found in a human-SARS-CoV (AY463060) strain of the late phase of the 2003 epidemic. Yuan *et al.* (2010) observed that this deletion probably occurred independently in Rs672 and Human-SARS-CoV, and was not acquired through homologous recombination (Ren *et al.*, 2006).

Spike protein and its role in host tropism

The S protein is the largest structural protein encoded by all coronaviruses; it mediates receptor association and fusion of the viral and cellular membranes, and is the major antigenic determi-
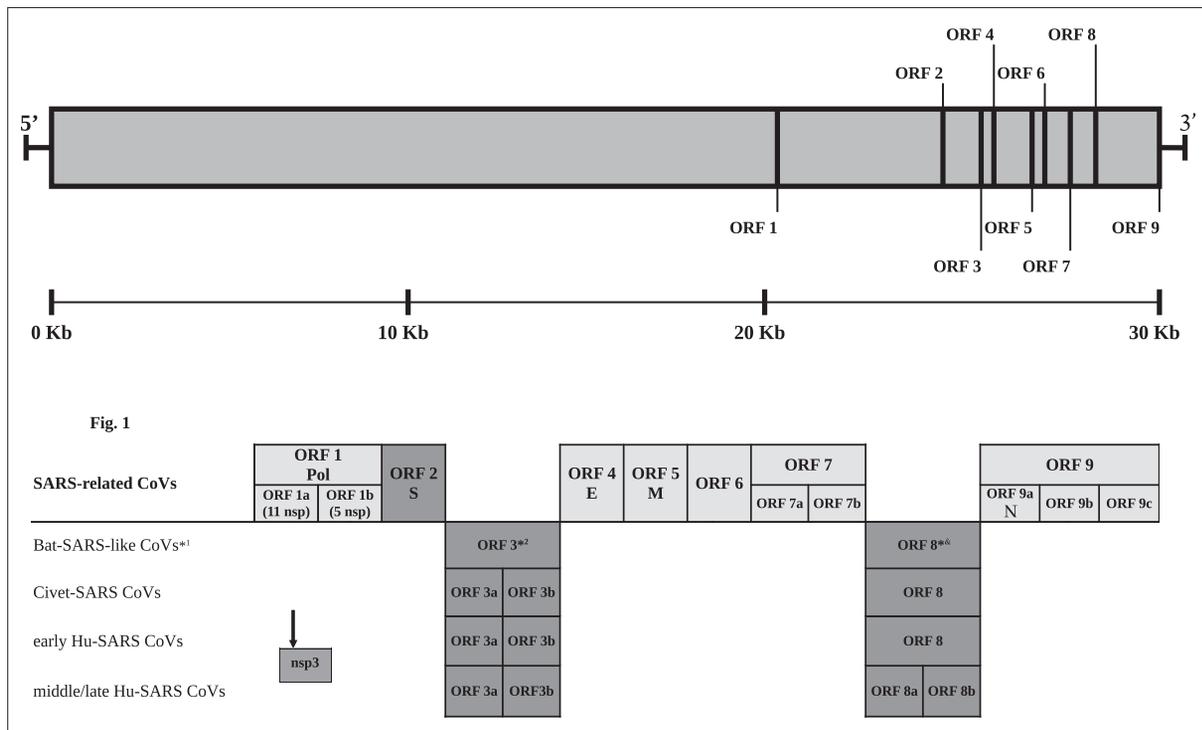

FIGURE 1A, B - *Typical genome organisation of human and civet SARS-CoVs and bat SARS-like-CoVs. 1a (upper panel) is a schematic diagram of SARS-related CoV genome organisation. 1b (lower panel) is a schematic representation of the diversities within the genome organisation of SARS-related CoVs. Light gray: little variable genome tracts, dark gray: highly variable genome tracts. *1: does not include African and European bat-SARS-like CoVs. *2: Rf1 strain presents ORF3a,b,c. *3: HKU3-8 strain presents ORF8a,b,c.*

nant of coronaviruses. The host range of a specific coronavirus is largely determined by its S protein which is composed of an N-terminal extremity (S1) for receptor binding and a C-terminal extremity (S2) for virus-cell fusion and entrance. (Li *et al.*, 2006).

The Receptor-Binding Domain (RBD) is a tract of about 200 amino acids of the S1 extremity with the function of linking to the cellular host receptor, the major immunodominant and potent neutralising epitope of the S protein. The RBD is composed of two sub-domains: a core and an extended loop. In the extended loop, there is the Receptor-Binding Motif (RBM) which directly contacts the cellular host receptor.

The S1 extremity of bat-SARS-like CoVs has a low degree of similarity to that of the SARS-CoV, especially in the RBD, whereas the S2 extremity has a high degree of similarity between the two viral groups. These differences imply that bat-SARS-like CoVs and the SARS-CoV made use of different molecules as entry receptors but have the same entry mechanism (Ren *et al.*, 2008; Shi *et al.*, 2008).

Human and civet angiotensin-converting enzyme 2 (ACE2) was identified as a functional cellular receptor for the SARS-CoV (expressed in the lung and the gastrointestinal tract of the hosts) whereas the SARS-like viruses found in bats are unable to use any of the human, civet and bat ACE2 molecules as their receptors and utilize another unknown receptor (Li *et al.*, 2006; Ren *et al.*, 2008; Graham *et al.*, 2010; Hou *et al.*, 2010; Yu *et al.*, 2010). This capability of SARS-CoVs to bind with human ACE2 is due to a minimal variation in the RBD sequence, and it is therefore possible that bat-SARS-like CoVs may attain this ability to infect human cells through the acquisition of an S sequence capable of binding to human ACE2. This event could occur by recombination with other CoVs if the same bat cells carry receptors for both types of viruses, one capable and one not capable of binding with ACE2 (Ren *et al.*, 2008).

This possibility is favoured by the greater genetic diversity of ACE2 among bats as opposed to that observed among known SARS-CoV-susceptible mammals (Hou *et al.*, 2010). This diversity has also pointed that many uncharacterized bat species may be able to act as reservoirs of the SARS-CoV or its progenitor viruses. Indeed, it has

already been shown that *Rhinolophus sinicus* and *Myotis daubentonii* ACE2s are also capable of supporting SARS-CoV entry, albeit with a different efficiency in comparison to that of human ACE2 (Hou *et al.*, 2010).

Possible African origin of the SARS-like CoV

To date, SARS-like CoVs have been detected in Asia, Europe and Africa. In Asia and Europe, the SARS-like CoVs are strictly related to some species of the *Rhinolophus* bat genus (Lau *et al.*, 2005; Li *et al.*, 2005; Woo *et al.*, 2006; Lau *et al.*, 2007; Woo *et al.*, 2007; Drexler *et al.*, 2010; Lau *et al.*, 2010; Rihtaric *et al.*, 2010; Yuan *et al.*, 2010; Balboni *et al.*, 2011;) whereas, in Africa, the SARS-like CoVs have been detected in several species of the *Hipposideros* and *Chaerophon* bat genera, indicating the lack of a strict species-specific host restriction in this geographical area (Pfefferle *et al.*, 2009; Tong *et al.*, 2009; Quan *et al.*, 2010).

The absence of a species-specific host restriction, which bat-SARS-like CoVs have shown only in the African area, is also supported by the previous detection of reactive antibodies against SARS-CoV antigens in sera belonging to seven different bat genera (*Hypsignathus*, *Lyssonycteris*, *Miniopterus*, *Mops*, *Myonycteris*, *Rhinolophus* and *Rousettus*) (Muller *et al.*, 2007)

Of considerable interest is ZBCoV (HQ166910), the SARS-like CoV strain detected by Quan *et al.* (2010) in Nigeria which presents several genome peculiarities in comparison to other SARS-like CoVs; the principal peculiarities are:

1) the TRS motif is not present in ORF6;
2) the putative S protein presents greater sequence differences as compared to other SARS- and SARS-like CoVs, especially in the S1 domain involved in receptor binding;
3) a short ORF3;
4) a significantly shorter region between the M and N genes, caused by a large deletion, with ORf6 which overlaps with the M gene, ORF7 which overlaps with ORF6 and is not subdivided into a and b, ORF8 which overlaps with ORF7 and the N gene;
5) the presence of two conserved s2m (instead of one) downstream from the N gene.

The ZBCoV is most closely related to the GhanaBt-CoVs detected by Pfefferle *et al.* (2009) in Ghana. Phylogenetically, these African strains

cluster together and could be considered a separate subgroup within the *Betacoronavirus*, in an ancestral position to the SARS-related CoV subgroup, which includes SARS-CoVs and the other Asian and European Rhinolophus SARS-like CoVs (Pfefferle *et al.*, 2009; Quan *et al.*, 2010). These findings could support the possible origin of the SARS-like CoVs in Africa rather than in Asia, and it is therefore possible to hypothesise that a subsequent migration occurred from Africa to Asia which may have led to the origin of the SARS-related coronavirus subgroup (Quan *et al.*, 2010).

The european scenario
Despite the large size and mild climate of the Mediterranean area, the European bat population is composed of a small number of species (Table 2), which contrasts with the high species diversity characterising the order *Chiroptera*. Bats are still present in all European countries, with the Mediterranean area having a major diversity. At present, there is still little information on the presence of coronaviruses in the European bat population.

Not many studies have been carried out and they have focused, for the most part, on central-east Europe, investigating only some of the species which make up the diversity of the European bat population.

Due to its characteristic climatic conditions and geographical position, the Mediterranean area is characterized by remarkable biodiversity and represents a strategic bridge for the viruses from the Middle East and Africa to cross to Europe. Nevertheless, with the exception of one preliminary study (Balboni *et al.*, 2011), the Mediterranean area has been ignored by most other studies.

The first investigation on the presence of coronaviruses in European bats was carried out in 2008 by Gloza-Rausch *et al.* (2008) in Northern Germany. Gloza-Rausch *et al.* tested faeces sampled from more than 300 bats by reverse transcription-polymerase chain reaction (RT-PCR); a high conservative tract was amplified for all the coronaviruses of the RNA-dependent RNA polymerase (RdRp, the 12th non structural protein codified to ORF1a,b) which is frequently used for the phylogenetic comparison of coronaviruses. Bat-CoVs belonging to the *Alphacoronavirus* were detected with an overall prevalence of 9.8%. All

the positive bats belonged to the *Myotis* and *Pipistrellus* bat genera, but no samples from the *Rhinolophus* spp. were included in the study. This study did not identify the coronavirus correlated with the SARS-CoV but hypothesised a close association of bat-CoVs with bat species rather than with sampling locations, and a central role of juvenile bats in virus amplification and transmission to adult females in maternity colonies.

In 2010, Reusken *et al.* (2010) carried out a study on the presence of coronaviruses in the Dutch bat population. More than 200 bats representing 13 different bat species were tested using RT-PCR for the presence of coronaviruses; a high percentage of infection (16,9%) was detected. This study found no SARS-related CoV but coronaviruses belonging to the *Alpha-* and *Betacoronavirus* were detected in some species of *Myotis*, *Nyctalus* and *Pipistrellus* bat genera and a closer association of the viruses with bat species than with sample location was observed. The 16.9% prevalence of coronavirus infection detected in the Netherlands was almost double that found in Northern Germany (9.8%), although some species tested were the same. These data might suggest a variation in the geographic distribution of bat-CoVs which could be influenced by several factors, such as the latitude and climate of the sampling area, the composition and density of the population, and different bat behavior and ecology.

Furthermore, both studies sampled two bat species belonging to the *Myotis* genus (*M. daubentonii* and *M. dasycneme*) more than the other species and, whereas *M. dasycneme* had approximately the same prevalence of infection in both studies, *M. daubentonii* had a far greater prevalence of infection in the Netherlands than in Northern Germany. Therefore, to confirm what has already been hypothesized, the divergence of the prevalence of infection between different European areas may be more related to the single species than to general environmental and geographical factors.

In 2010-2011, three other studies on coronaviruses in bats in different European countries were published (Drexler *et al.*, 2010; Rihtaric *et al.*, 2010; Balboni *et al.*, 2011). In these studies, the study sample also included faeces belonging to bats of the *Rhinolophus* spp. which, in Europe, is represented by five different species having dif-

ferent territorial distributions (Table 2 and Fig. 2a-e) (*www.faunaeur.org*). These studies demonstrated a strict correlation between SARS-like CoVs and *Rhinolophus* spp. bats in Europe as has also been shown in Asia.

Rihtaric *et al.* (2010) carried out a study on the presence of CoVs in bats in Slovenia, amplifying a highly conservative fragment of the RdRp by RT-PCR. Fourteen of the 106 bats analyzed were positive and all belonged to the 36 *Rhinolophus*

TABLE 2 - *Bat species present in Europe*

European bat species (www.faunaeur.org)

Suborder	Family	Genus and species	IT	ES	FR	GE	GB	NL	BG	SL
Macrochiroptera	*Pteropodidae*	*Rousettus aegyptiacus*								
Microchiroptera	*Molossidae*	*Tadarida teniotis*	X	X	X				X	
	Rhinolophidae	*Rhinolophus ferrumequinum*	X	X	X	X	X		X	X
		Rhinolophus hipposideros	X	X	X	X	X		X	X
		Rhinolophus euryale	X	X	X				X	X
		Rhinolophus blasii	X	X	X				X	
		Rhinolophus mehelyi	X	X	X				X	
	Vespertilionidae	*Barbastella barbastellus*	X	X	X	X	X		X	X
		Eptesicus botae								
		Eptesicus nilssonii	X		X	X			X	X
		Eptesicus serotinus	X	X	X	X	X	X	X	X
		Hypsugo savii	X	X	X				X	X
		Miniopterus schreibersii	X	X	X				X	X
		Myotis alcathoe		X	X					
		Myotis aurascens	X						X	
		Myotis bechsteinii	X	X	X	X	X	X	X	X
		Myotis blythii	X	X	X				X	X
		Myotis brandtii	X		X	X	X	X	X	X
		Myotis capaccinii	X	X	X				X	X
		Myotis dasycneme	X		X	X		X	X	
		Myotis daubentonii	X	X	X	X	X	X	X	X
		Myotis emarginatus	X	X	X	X		X	X	X
		Myotis myotis	X	X	X	X	X	X	X	X
		Myotis mystacinus	X	X	X	X	X	X	X	X
		Myotis nattereri	X	X	X	X	X	X	X	X
		Myotis punicus	X		X					
		Nyctalus azoreum								
		Nyctalus lasiopterus	X	X	X				X	
		Nyctalus leisleri	X	X	X	X	X	X	X	X
		Nyctalus noctula	X	X	X	X	X	X	X	X
		Pipistrellus kuhlii	X	X	X	X			X	X
		Pipistrellus maderensis		X						
		Pipistrellus nathusii	X	X	X	X	X	X	X	X
		Pipistrellus pipistrellus	X	X	X	X	X	X	X	X
		Pipistrellus pygmaeus	X	X	X	X	X		X	X
		Plecotus auritus	X	X	X	X	X	X	X	X
		Plecotus austriacus	X	X	X	X	X	X	X	X
		Plecotus kolombatovici								
		Plecotus macrobullaris	X	X	X					X
		Plecotus sardus	X							
		Plecotus teneriffae		X						
		Vespertilio murinus	X		X	X	X	X	X	X

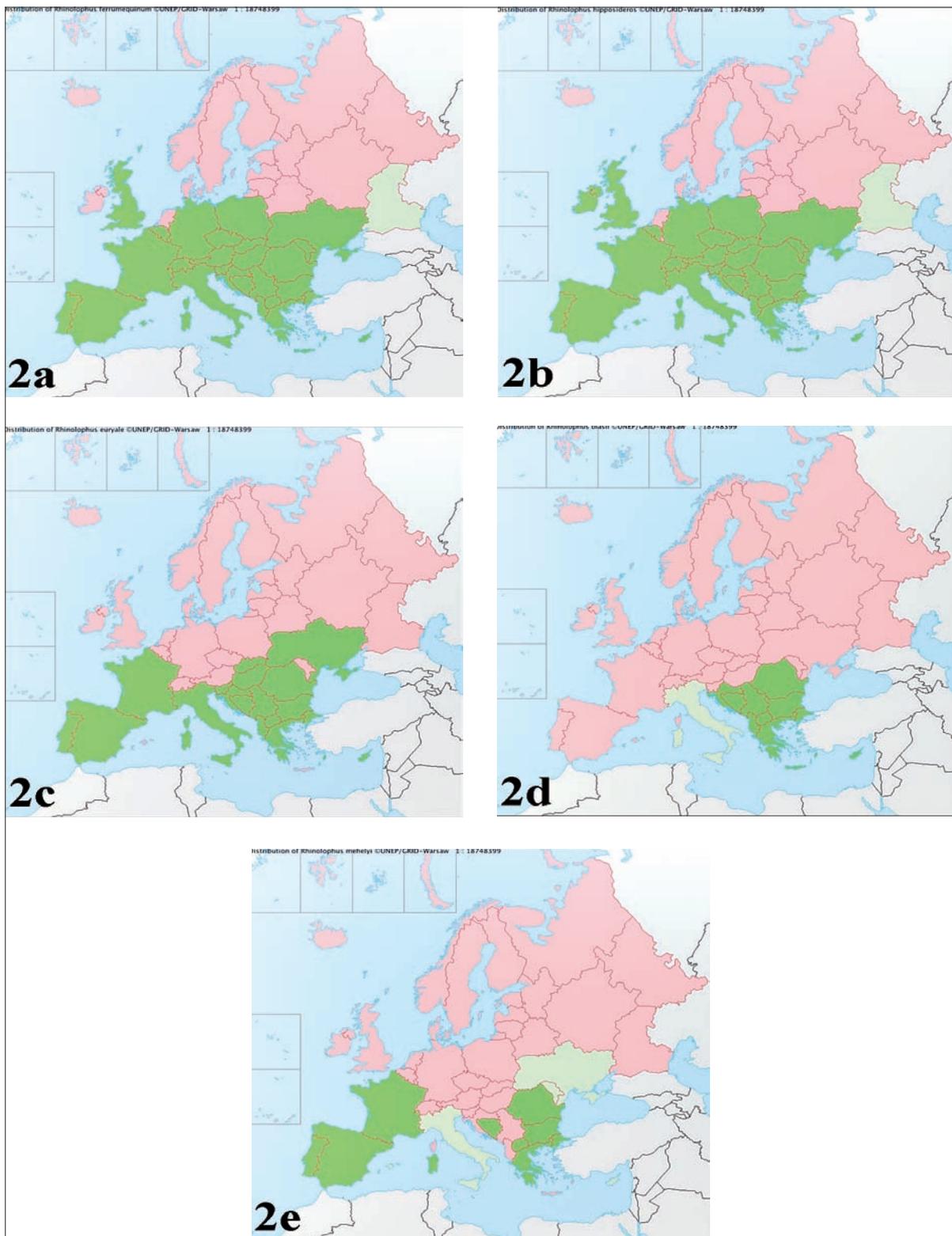IT=Italy; FR=France; GB=United Kingdom; ES=Spain; GE=Germany; NL=Netherlands; BG=Bulgaria; SL=Slovenia.

FIGURE 2 - *European distribution of the Rhinolophus bat species (www.faunaeur.org): 2a: R. ferrumequinum; 2b: R. hipposideros; 2c: R. euryale; 2d: R. blasii; 2e: R. mehelyi. GREEN: present. PALE GREEN: doubtful. RED: absent.*

hipposideros bats included in the population sample. Only the *Rhinolophus* spp. was positive to coronavirus infection, and bioinformatic analysis showed that the Slovenian bat coronaviruses belonged to the *Betacoronaviruses* and that the most closely related virus sequence in GenBank was SARS bat Rp3 isolated from *Rhinolophus sinicus* in China in 2004 (Li *et al.*, 2005; Rihtaric *et al.*, 2010; Yuan *et al.*, 2010). As in the first German study, the positive bats were young bats or lactating females, corroborating the hypothesis that the virus replicates more efficiently in young bats than in adults, and that young bats transmit the virus to adult females in maternity colonies.

In Rihtaric *et al.'s* study, 26 *Myotis daubentonii* bats were tested and were all negative.

Considering what had already been seen in the Netherlands and Northern Germany for this species, there may be a progressive increase in the prevalence of coronavirus infection proceeding from southeast to northwest Europe.

The research of Balboni *et al.* (2011) on bat CoVs involved only one species of the *Rhinolophus* genus found in the Italian peninsula, the *R. ferrumequinum*.

They utilized the same method as that used in preceding studies, an RT-PCR amplifying a highly conserved fragment of RdRp, detecting two positives of the 52 bats sampled, with a virus prevalence of 3.8%. Sequence analysis of one strain showed that it was genetically closely related to SARS- and other SARS-like coronaviruses belonging to the *Betacoronavirus*.

Furthermore, the Italian strain showed a close correlation to the Slovenian strains whereas the similarities with the African strain were low.

Drexler *et al.* (2010) conducted an evaluation of CoVs in rhinolophid and vespertilionid bat species in Bulgaria, in an area in which all five European *Rhinolophidae* species coexist. Two hundred and two of the 499 bats were positive with RT-PCR or nested RT-PCR when amplifying a highly conserved fragment of RdRp. The viruses detected were either *Alpha-* and *Betacoronavirus*, but all the SARS-like CoVs occurred exclusively in *Rhinolophus* (101 positives out of 389 *Rhinolophus* bats sampled).

A Bulgarian SARS-like virus was fully sequenced and its genome showed some special features in comparison with other known SARS-like CoVs, namely:

1) the high divergence of predicted proteins 3b and 6;
2) the absence of ORF8;
3) a greater similarity of the Bulgarian SARS-like virus spikes RBD to SARS-CoVs than to the other bat-SARS-like CoVs.

From the research carried out to date, it can be hypothesized that CoVs are widely diffused in European bats and that SARS-like CoVs are present in close correlation to bat species belonging to the *Rhinolophus* genus, as has been noted in Asia (Tang *et al.*, 2006; Cui *et al.*, 2007; Lau *et al.*, 2007; Shi *et al.*, 2008).

The full genome sequence obtained in one Bulgarian bat-SARS-like CoV (GU190215) (Drexler *et al.*, 2010) also has some special features which distinguish it from the Asian and the African strains.

The total absence of ORF8 is an unexpected deletion for a bat-SARS-like-CoV genome since almost all SARS-related coronavirus strains detected in the *Rhinolophus* spp were characterized by a single ORF8, with the insertion of an 29-nts sequence which fuses together the ORF8a and 8b distinctive for Human-SARS-CoV. If it were hypothesized that the 29 nts deletion in the ORF8 of the Human-SARS-CoV represented a greater functional hallmark in the transition of the SARS-CoV to humans (Lau *et al.*, 2005; Oostra *et al.*, 2007), the absence of this ORF in the European SARS-like CoV would suggest that protein 8 may not, in general, be essential for the maintenance of the SARS-like CoV in bats (Drexler *et al.*, 2010).

This peculiar deletion in the Bulgarian SARS-like CoV, in association with the high nucleotide variability of some genes and the greater similarity of spikes RBD with the SARS-CoV than with other bat-SARS-like CoVs, suggests a possible divergence between the Asian bat SARS-like CoVs and the European bat SARS-like CoVs.

This assumption requires more extensive investigation on the European bat population to acquire more information on the epidemiology of bat-SARS-like CoVs on this continent.

The studies should also focus more closely on the bat species belonging to the *Rhinolophus* spp. and on the epidemiological situation in the Mediterranean area which could play an important role in the evolution of these viruses.

DISCUSSION

In recent decades, with the emergence of several zoonoses which have had bats as animal reservoirs, numerous studies together on the central role of bats in the dissemination of zoonotic agents have been carried out. Among these, a high variability of coronavirus strains has been discovered in several species of bats in Asia, North and South America, Africa and Europe after the SARS epidemic in the past ten years.

All coronaviruses found in bats belong to two of the three coronavirus genera: *Alphacoronavirus* and *Betacoronavirus*. Of particular importance, with regard to the epidemiology of SARS-CoV, is a group of coronaviruses found in some species of bats and belonging to the *Betacoronavirus*, the bat-SARS-like coronaviruses. These viruses are genetically closely related to the SARS-CoV and are probably the ancestral viruses of the marketplace animal- and human- SARS-CoVs which caused the human epidemic in 2002-2003. To date, bat-SARS-like CoVs have been discovered in Asia, Africa and Europe. Taking into account that there have been many more studies carried out on the Asian region than in Africa and Europe, some differences can be pointed out regarding these three continents.

First, the host range of bat-SARS-like CoVs is limited to the *Rhinolophus* bat genus in both Asia and Europe, suggesting a close species-specific relationship. Instead, bat-SARS-like CoVs were found in numerous different bat species belonging to several genera.

Second, numerous genomic features differentiating the coronaviruses belonging to the three continents have been added to the host differences. Thus, if it is reasonable to think that the SARS-CoV causing the human epidemic derived from the SARS-like CoVs found in Asian bats, the theories regarding the area of origin and the phylogenetic evolution of these viruses are more controversial.

A possible origin of the SARS-like CoVs in Africa was hypothesized by Quan et al. (2010) in the light of the characteristics of some strains, such as the ZBCoV and GhanaBt-CoVs (Pfefferle et al., 2009; Quan et al., 2010) identified in Nigeria and Ghana, respectively, which could form a separate subgroup distinct from Asian and European Rhinolophus SARS-related CoVs and could be traced back to a common ancestor with the SARS-related CoV subgroup.

The African origin may have been followed by a migration which brought the bat-SARS-like CoVs to adapt to bats belonging to the *Rhinolophus* spp., thus arriving in Asia and Europe.

It is unclear whether the arrival of the bat-SARS-like CoVs in Europe followed the viral adaptation in Asian bats, or whether these viruses were first colonized in Europe, via the Mediterranean area, and from there were then passed on to Asia. On the other hand, the arrival of SARS-like CoVs from Africa to European and Asian bats could also have been the result of two independent events, with the origin of distinct viral populations, rather than two consecutive events.

Some special features of the Bulgarian SARS-like CoV distinguish it from the Asian and African strains (Drexler et al., 2010), but only one completely sequenced European strain is not sufficient to clarify the evolution and the migratory events which could have characterized these viruses in Europe.

More detailed studies on the European bat population with particular reference to the Mediterranean area are required to clarify these aspects of the bat-SARS-like CoV evolution and the ramifications concerning public health as regards the potential of coronaviruses to cross species barriers and thus infect humans, as people come into closer contact with wild animals.

In this regard, and to better assess the prevalence of infection of coronaviruses in bats, the development and use of diagnostic techniques with high sensitivity is important. Indeed, the principal diagnostic method used by various researchers, especially during the initial screening phase of the population sample, is a reverse transcription-PCR (RT-PCR) amplifying a fragment of variable size of the RNA-dependent RNA polymerase gene (RdRp, the 12th non structural protein codified to ORF1a, b), which is a genome tract frequently used for the molecular detection and phylogenetic analysis of bat coronaviruses (Lau et al., 2005; Lau et al., 2007; Woo et al., 2007; Lau et al., 2010; Rihtaric et al., 2010; Yuan et al., 2010; Balboni et al., 2011). However, this method may not have optimal sensitivity, causing an underestimation of the true prevalence of infection. This may be due to two main factors: first, bats are reservoirs of coronaviruses and may shed on

a small amount of viruses in faeces, too low to be detected by traditional PCR methods; second, the RdRp, although highly conserved in coronaviruses, has a moderate degree of nucleotide variability which can result in a sub-optimal match of the primers used.

In the near future, the tactics used by humans to prevent the spread of bat-associated zoonosis, should be based on two main lines of action: on the one hand systematic surveillance with appropriate techniques aimed at controlling known pathogens, such as the bat-SARS-like CoV, and discovering possible new zoonotic agents; on the other hand, the conservation of the natural habitats of bats, thus avoiding direct contact with humans and livestock.

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