Circulation of Group 2 Coronaviruses in a Bat Species Common to Urban Areas in Western Europe

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Abstract

Fecal samples of 211 bats representing 13 different bat species from 31 locations in the Netherlands were analyzed for the presence of coronaviruses (CoV) using a genus-wide reverse transcription (RT)–polymerase chain reaction. CoVs are known for their high potential for interspecies transmission, including zoonotic transmission with bats as reservoir hosts. For the first time, a group 2 CoV was found in a bat, *Pipistrellus pipistrellus*, in Europe. This is of particular interest for public health as the reservoir host is a species that is common to urban areas in most of Europe and notorious for its close interactions with humans. Four verspertilionid bat species were found to excrete group 1 CoVs, viz. *Myotis daubentonii*, *M. dasycneme*, *P. pipistrellus*, and *Nyctalus noctula*. The last species is a newly identified reservoir. The overall prevalence was 16.9% and positive bats were found at multiple widespread locations. The circulating group 1 CoV lineages were rather species associated than location associated.

Key Words: Bats—Coronavirus— Field study—Public health—Zoonosis.

Introduction

THE MAJORITY OF EMERGING INFECTIOUS diseases in humans are zoonotic and emerging from wildlife reservoir hosts. Frequently, these zoonoses are caused by RNA viruses (Jones et al. 2008). Recent examples are the influenza A (H1N1) pandemic in 2009 (Dawood et al. 2009) and the outbreak of severe acute respiratory syndrome (SARS) in 2002–2003, caused by the novel coronavirus SARS-CoV (Drosten et al. 2003). The genus coronavirus (CoV) belongs to the family of the Coronaviridae in the order of the Nidovirales. CoVs are enveloped, positive-sense RNA viruses with large genomes (29–32 kb) packaged in particles with corona-like morphology (Holmes 2001). CoVs comprise three genetically and serologically distinct groups (Gonzalez et al. 2003). Group 1 and 2 viruses are pathogenic for mammals. Group 1 includes human CoV-229E (hCoV-229E) and hCoV-NL63. Group 2 includes hCoV-OC43 and hCoV-HKU1. Group 3 viruses are foremost pathogenic for poultry.

CoVs are considered as a group of viruses with a high potential for interspecies transmission. They are found in a wide variety of hosts, including wildlife, livestock, poultry, pets, and humans. Due to their long genomes, high recombination frequency and high mutation rate, CoVs have the potential to adapt to new host species (Woo et al. 2009). Examples of interspecies transmission of CoVs are the emergence of porcine epidemic diarrhea CoV in swine in Europe most likely from bats (Pensaert and de Bouck 1978, Vijaykrishna et al. 2007), hCoV-OC43, which is believed to be the result of a zoonotic transmission event of bovine CoV (Vijgen et al. 2005), and SARS-CoV, which was shown to be introduced into the human population from civet cats, which acted as intermediate hosts, whereas bats are considered as the primary reservoir hosts (Lau et al. 2005). Very recently, evidence was found for a 208–322-year-old common ancestor for a group 1 CoV and hCoV-229E circulating in *Hipposideros* bats in Ghana (Pfefferle et al. 2009).

A large number of viruses have been detected in bats. However, zoonotic transmission has only been shown for rabies virus, bat lyssa viruses, Melaka reovirus, Ebola virus, Marburg virus, Nipah and Hendra viruses, and SARS-like CoV (Calisher et al. 2006, Wong et al. 2007). Bats (order *Chiroptera*) constitute around 20% of all mammalian species and are geographically widely dispersed with a wide variety of habitats (Jones et al. 2002). Bats have a number of characteristics that increase the likelihood of intra- and interspecies transmission of viruses. They have, in general, a long life span, they fly daily in pursuit of food, and some species fly long

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distances during seasonal migrations. Further, they show gregarious roosting behavior, with up to several millions of individuals per population. Roost sites include locations in proximity to humans like houses (cellars and cavity walls), stables, bridges, mines, and wells; frequent contact with intermediate hosts like domestic cats enlarge the possibility of transmission of virus from bats (Calisher et al. 2006). Recent studies indicate that bats are the natural hosts for all mammalian CoVs and that the circulation of CoVs in other mammals is the result of occasional introductions from bats

(Vijaykrishna et al. 2007, Woo et al. 2009). Genetic analyses show that bat CoVs belong to groups 1 and 2. Within group 2 they cluster in the subgroups 2b, which includes all SARS and SARS-like CoV from different hosts, and 2c and 2d, which represent two independent bat CoV subgroups.

To assess the putative risks for public health of bats as reservoirs for CoVs with potential for interspecies transmission, we investigated to what extent CoVs circulate in a variety of bat species and populations in the Netherlands. Isolated bat CoV sequences were sequenced and analyzed
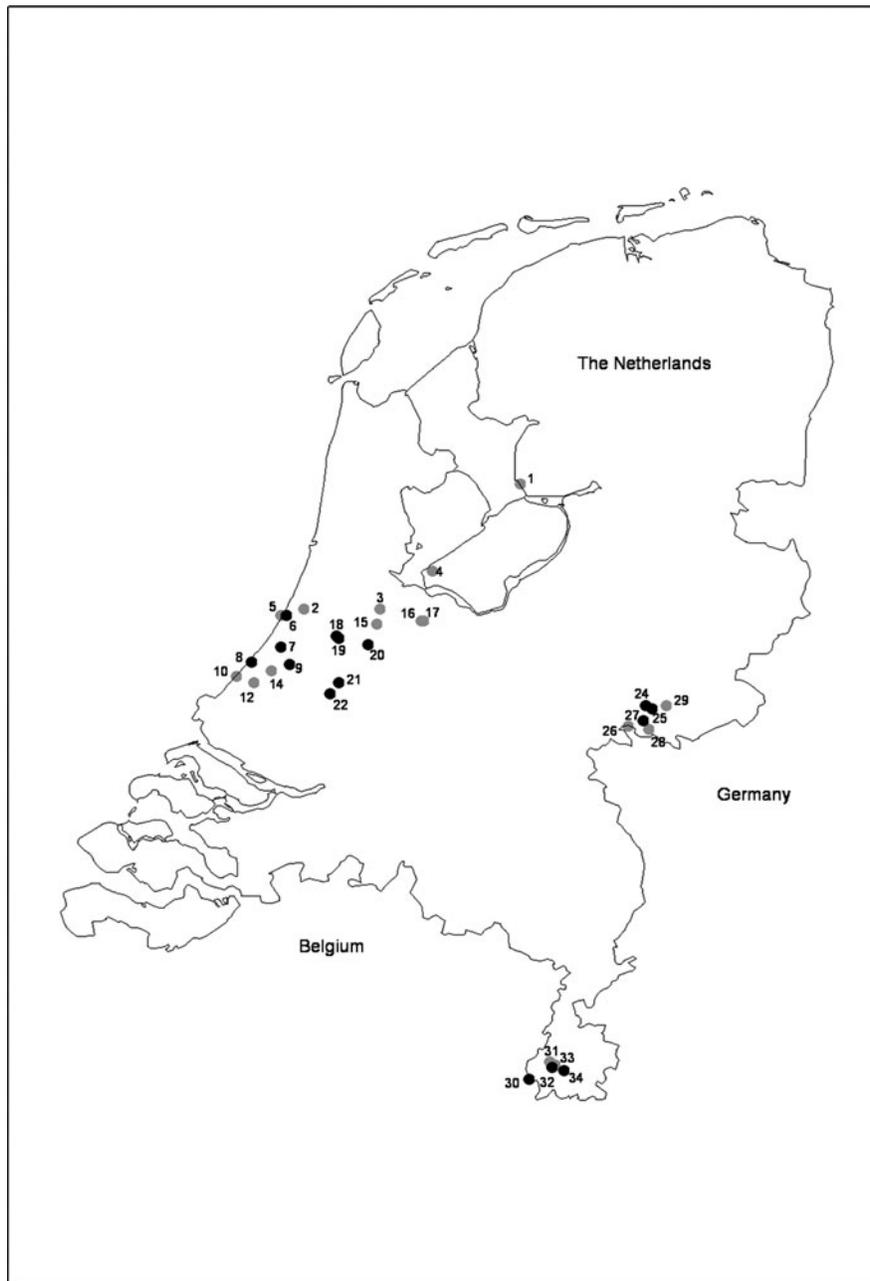
FIG. 1. Distribution of sampled locations (a gray dot) and coronavirus (CoV)-positive locations (a black dot). Sampling locations are indicated with a unique number: 1, Tollebeek; 2, Lisse; 3, Stokkelaarsbrug; 4, Almere; 5, Noordwijk aan Zee; 6, Noordwijkerhout; 7, Wassenaar; 8, Scheveningen; 9, Stompwijk; 10, Den Haag; 12, Rijswijk; 14, Leidschendam; 15, Waversveen; 16,'s Gravenland; 17,'s Gravenland; 18, Papenveer; 19, Ter Aar; 20, Woerdense verlaat; 21, Reeuwijk; 22, Gouda; 24, Doetinchem–De Huet; 25, Doetinchem–Wijnbergen; 26, Veldhuizen; 27, Beekermark-Zeddam; 28, Zeddam; 29, Doetinchem–Slangenburg; 30, Maastricht-west; 31, Meerssen; 32, Berg en Terblijt; 33, Geulhem; 34, Sibbe.

phylogenetically. To our knowledge this is the first report of group 2 CoV circulation in bats in Europe. We show that group 1 CoVs are commonly present in a variety of bat species at multiple locations in the Netherlands. This includes the identification of a new reservoir species.

Materials and Methods

Sampling

Bats were caught in several regions of the Netherlands (Fig. 1). With exception of a few grounded bats, bats were caught with mist nets in their foraging habitats in forests or over water bodies (Haarsma and van Alphen 2009), and in the southernmost part of the country at swarming sites at the entrances of subterranean limestone quarries. After catching, each bat was kept in a disposable cotton bag for the collection of fecal pellets, after which the species, sex, age, and reproduction status were determined. All bats were released at their capture site. The feces were collected with disposable tweezers and stored at −20°C till processing. All procedures were in compliance with Dutch laws on animal handling and welfare and wildlife conservation (Flora and Fauna Act license FF/75A/2003/169/a/b; Animal Ethics Clearance 802-18230-00/2006025).

RNA isolation from bat feces

Feces were resuspended in HBSS HANKS medium (Invitrogen, Breda, The Netherlands). In accordance to specimens availability, 10%, 5%, or 1% of fecal suspensions were used. The fecal suspensions were centrifuged at 3000 g for 20 min, and 100 μL supernatant was used for extraction of RNA. RNA was extracted as previously described (Boom et al. 1990). To check for cross-contamination, a negative control sample was included for every four samples.

Reverse-transcription polymerase chain reaction and sequencing

A nested CoV genus-wide reverse transcription (RT)-polymerase chain reaction (PCR) was performed as described previously (de Souza Luna et al. 2007). To exclude false-negative results due to the presence of RT-PCR inhibiting factors, each sample was also analyzed in the presence of a CoV spike RNA at an experimentally predetermined detectable concentration. Only the results of samples that were positive for CoV in the spiked RT-PCR were taken into account in this study. PCR amplicons were sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Applied Biosystems, Nieuwerkerk a/d, Ijssel, The Netherlands). All sequences were confirmed by sequencing both strands. Sequences were edited using Kodon vs 3.5 (Applied Maths, St–Martens–Latem, Belgium).

Phylogenetic analysis

Nucleotide sequences were analyzed using Bionumerics vs 5.1 (Applied Maths). Evolutionary trees of nucleotide sequences were drawn using the maximum parsimony method. The confidence values of the internal lineages were calculated by performance of 1000 bootstrap analysis. Sequences from the Dutch bat-CoVs are submitted in GenBank under accession nos. GQ259960-GQ259977. Accession numbers used in the analysis are as follows: AY427798 (Toro virus); EU420138 (*Miniopterus magnater*, China, 1A); EF203064 (*Rhinolophus sinicus*, China, HKU2); EU375860 (*Myotis dasycneme*, Germany, D3.10); EU375855 (*M. dasycneme*, Germany, D3.4); EU375855 (*Pipistrellus nathusii*, Germany, D5.16); EU375870 (*Pipistrellus pygmaeus*, Germany, D5.85); EU375874 (*Myotis daubentonii*, Germany, D8.38); EU375872 (*M. daubentonii*, Germany, D8.45); DQ249224 (*Myotis ricketii*, China, HKU6); NC_002645 (hCoV-229E); NC_005831 (hCoV-NL63); DQ648854 (*Rhinolophus* spp., China, A970); EU834953 (*Rhinolophus megaphyllus*, Australia, 100); DQ249226 (*M. magnater*, China, HKU7); NC_010438 (*Miniopterus pusillus*, China, HKU8); AY597011 (hCoV-HKU1); NC_005147 (hCoV-OC43); AY641576 (Avian infectious brochitis virus); EF065515 (*Rousettus lechenaulti*, China, HKU9/3); EF065514 (*R. lechenaulti*, China, HKU9/2); DQ648856 (*Rhinolophus ferrumequinum*, China, 273); DQ022305 (*R. sinicus*, China, HKU3); DQ648857 (*Rhinolophus macrotis*, China, 279); AY304488 (Civet SARS-CoV-SZ16); DQ071615 (*Rhinolophus pearsoni*, China, Rp3); EF065505

TABLE 1. OVERVIEW OF BAT SPECIES TESTED FOR CORONAVIRUSES

Species	Total no. (positive)	No. of male adult/juvenile (positive)	No. of female adult/juvenile (positive)	No. of female lactating/gravid (positive)	Locations[a]
Eptesicus serotinus	1 (0)	0 (0)/1 (0)			14
Myotis bechsteinii	4 (0)	4 (0)/0 (0)			30
Myotis brandtii	2 (0)	1 (0)/0 (0)	1 (0)		26, 32
Myotis daubentonii	50 (8)	17 (4)/2 (0)	20 (2)/2 (0)	9 (2)	6, **9**, 12, 15, 20, **27**, **30**, **32**, 33, **34**
Myotis dasycneme	105 (20)	13 (1)/21 (7)	48 (9)/18 (3)	5 (0)	1, 3, 4, **9**, 15, **18**, **19**, **20**, **21**, **22**
Myotis emarginatus	6 (0)	5 (0)/0 (0)	1 (0)		30, 31, 32, 34
Myotis myotis	1 (0)	1 (0)/0 (0)			31
Myotis mystacinus	3 (0)		3 (0)		26, 28, 29
Myotis nattereri	2 (0)		2 (0)		26, 31
Nyctalus noctula	14 (5)	5 (2)/0 (0)	8 (2)	1 (1)	**6, 7, 8**, 10
Pipistrellus nathusii	8 (0)	8 (0)/0 (0)			2, 5, 6, 7
Pipistrellus pipistrellus	8 (2)	1 (1)/1 (1)	3 (0)	3 (0)	6, 17, **24**, **25**
Plecotus auritus	7 (0)	3 (0)/0 (0)	3 (0)	1 (0)	16, 17, 26, 27, 29, 31

[a]Numbers of locations with CoV-positive bats are depicted in bold. Distribution locations in the Netherlands are shown in Figure 1.
CoV, coronavirus.

TABLE 2. ESTIMATED PREVALENCES OF CORONAVIRUS
IN FOUR POSITIVE BAT SPECIES IN THE NETHERLANDS

| | | 95% CI | |
	CoV prevalence	Lower	Upper
Overall	16.9%	12.2%	22.2%
Species			
M. dasycneme	19.6%	12.7%	27.6%
M. daubentonii	17.3%	8.4%	28.6%
N. noctula	37.5%	16.3%	61.6%
P. pipistrellus	30.0%	7.5%	60.0%

CI, confidence interval.

TABLE 3. PREDICTIVE FACTORS FOR CORONAVIRUS
DETECTION IN BATS IN THE NETHERLANDS

| | | | 95% CI | |
Predictive factor	Category	CoV prevalence	Lower	Upper
Sex	Female	15.4%	9.7%	22.0%
	Male	20.0%	12.3%	29.1%
Age	Adult	14.9%	9.9%	20.6%
	Female	15.4%	8.8%	23.4%
	Female lactating	19.0%	5.7%	37.9%
	Male	15.0%	7.2%	25.0%
	Juvenile	25.5%	14.3%	38.8%
	Female	18.2%	5.4%	36.3%
	Male	33.3%	17.2%	51.8%

(*Tylonycteris pachypus*, China, HKU4); EF065509 (*Pipistrellus abramus*, China, HKU5-1); EF065505 (*P. abramus*, China, HKU5-5); DQ648819 (*Pipistrellus pipistrellus*, China, A434); DQ648816 (*Pipistrellus* spp., China, 427); DQ648846 (*Pipistrellus* spp., China, A906); DQ648809 (*P. abramus*, China, 355); DQ648811 (*Pipistrellus* spp., China, 363A).

Statistics

A number of bats (*n*) were sampled independently at random (from the population of all bats at a location), such that in the sampling method each bat had the same probability (*p*) of being a bat infected with CoV. The prevalence (*p*) of CoV in (sub)populations of bats over selected locations in the Netherlands was estimated assuming this binomial process, where *p* can be expressed as the total number of positive bats (*k*) divided by *n*. The uncertainty about the estimated prevalence, due to sampling variability, is expressed using a beta distribution (Vose 2000).

Results

Bat surveillance and CoV detection

Fecal samples were collected from a total of 265 bats and analyzed for the presence of CoVs. Fifty-four bat samples were omitted from the study due to the presence of RT-PCR inhibiting factors. The remaining 211 samples represented 13 different bat species from 31 locations in the Netherlands (Table 1 and Fig. 1). In total, 78.7% of the sampled bats were adults. Females constituted 60.6% of the sampled bats, of which 15.6% were juvenile and 14.8% were lactating adults. Sixty-nine percent of the males were adults (Table 1).

The overall presence of CoVs in the bats was estimated at 16.9% (95% confidence interval = 12.2–22.2). Four vespertilionid bat species were found positive for CoV, viz. *M. daubentonii*, *M. dascyneme*, *Nyctalus noctula*, and *P. pipistrellus*, with estimated prevalences of 17.3%, 19.6%, 37.5%, and 30.0%, respectively. No significant differences in the estimated prevalences between the four bat species could be observed (Table 2).

At 15 of the 31 locations (48.4%) bats were found positive for CoVs. *M. daubentonii* was captured at 10 locations, of which 5 yielded CoV-positive bats (50.0%). Respectively, 60.0% (6/10) and 50.0% (2/4) of the locations sampled for *M. dasycneme* and *P. pipistrellus* were positive (Table 1). One location (location 9) harbored both CoV-positive *M. daubentonii* and *M. dasycneme* bats. No significant differences between locations per species were observed (data not shown).

The presence of phenotypic properties predictive for CoV infection in the Netherlands was investigated. None of the analyzed factors (sex, age, and lactation/gravidity) were predictive for CoV positivity—neither within the overall sampling population (Table 3), nor within subpopulations based on bat species (data not shown).

Group 1 and 2 CoVs in Dutch bats

The sequences of the PCR amplicons of 18 bat samples could be determined and were analyzed phylogenetically. Nucleotide sequence analysis showed that the bat-CoVs corresponding to the sequences amplified from Dutch bats are members of group 1 and 2 CoVs (Fig. 2A, B, respectively). Within group 1 five different lineages could be distinguished, bringing the total number of group 1 lineages for European bat-CoVs to 7 (Gloza-Rausch et al. 2008). Bat-CoV sequences isolated from *M. dasycneme* formed two distinct clades within group 1 (lineages 1 and 7). Each lineage was found at more than one location, and at location 22 (Fig. 1) representatives of both lineages were detected. The lineage 1 viruses clustered together with bat-CoVs isolated from *M. dasycneme* in Germany. Bat-CoVs associated with *M. daubentonii* clustered together regardless of the sample locations and in a clade with bat-CoVs isolated from the same bat species in Germany

FIG. 2. Phylogenetic analysis of CoV sequences found in bats in the Netherlands. Analysis based on a 334 bp fragment of the RNA-dependent-RNA-polymerase as described before (Gloza-Rausch et al. 2008). Numbers at the nodes indicate bootstrap values as percentage. Only values >70 are depicted. For all bat-CoVs putative reservoir species, country of origin, and identification number are depicted. Common human CoVs are included for reference; Breda torovirus was used as outgroup. CoVs identified in the present study are depicted in bold with the sampling location indicated between brackets. **(A)** Phylogenetic analysis of group 1 Dutch bat-CoVs. The various phylogenetic groups and lineages within group 1 are indicated on the right. **(B)** Phylogenetic analysis of group 2 Dutch bat-CoVs. The various phylogenetic groups and subgroups within group 2 are indicated on the right.
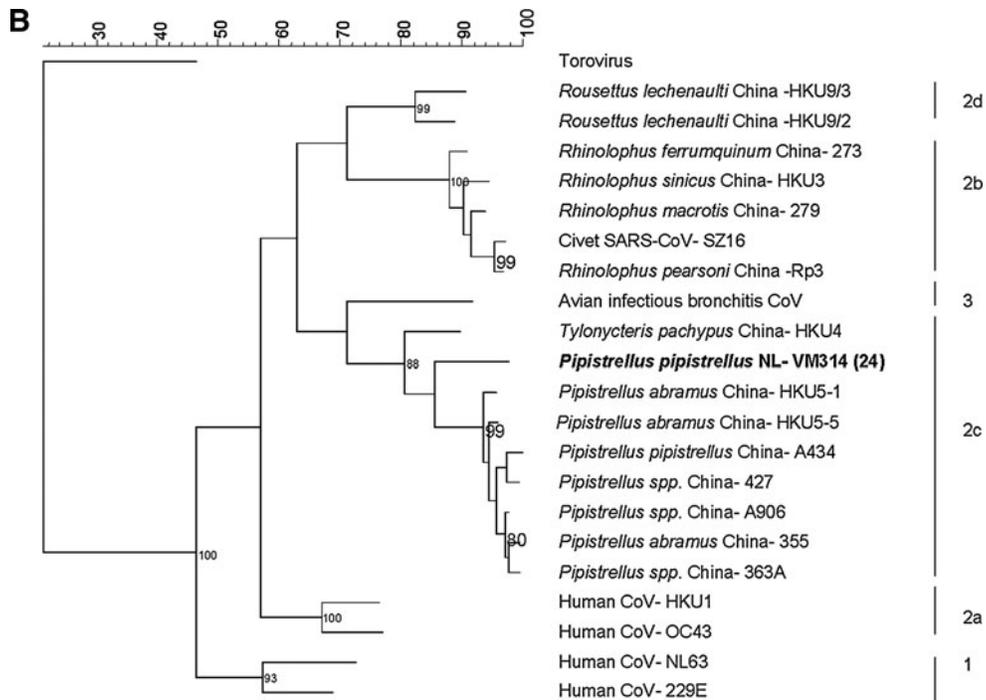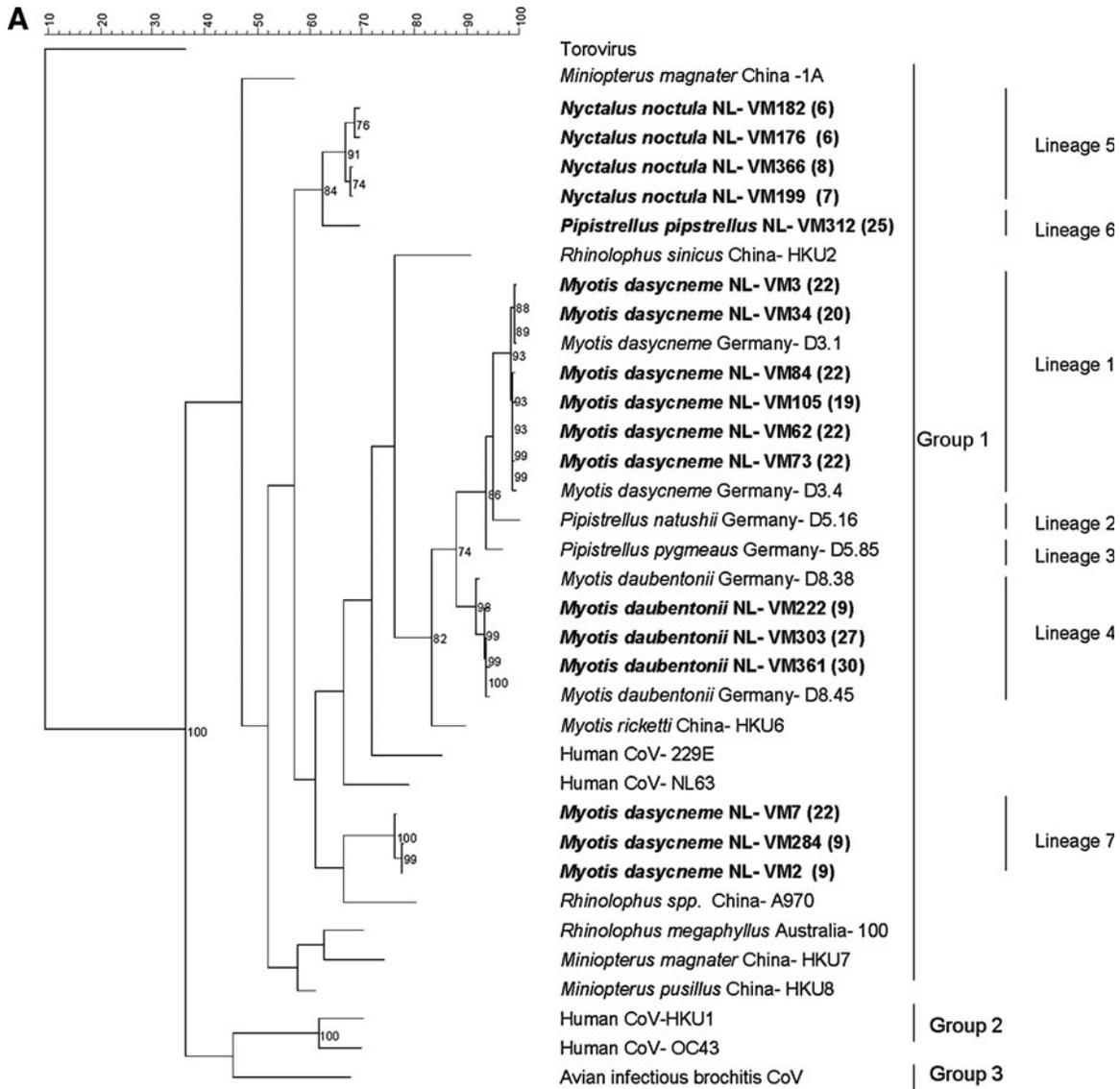
(lineage 4). Bat-CoVs associated with *N. noctula* captured at three different locations clustered together as well (lineage 5). These data support the Chinese and German observations that bat-CoVs are more closely associated with bat species than with sample locations (Tang et al. 2006, Gloza-Rausch et al. 2008). Both a group 1 (location 25) and a group 2 (location 24) CoV was found to be associated with *P. pipistrellus*. The group 1, lineage 6, CoV is most closely related to the lineage 5 viruses associated with *N. noctula*, the group 2 CoV clusters in putative subgroup 2c together with bat-CoVs isolated in China (Fig. 2B). The nucleotide identities with the Asian bat-CoVs in group 2c ranged from 64.96% to 97.57%. The closest relationship is found with CoVs associated with two Asian *Pipistrellus* species, viz. *P. pipstrellus* and *P. abramus.* The nucleotide identities between *Pipistrellus* spp. associated CoVs range from 79.22% to 97.57%.

M. dasycneme–associated Bat-CoV sequences within group 1 lineages 1 and 7 share nucleotide identities ranging from 97.61% to 99.71% and 97.63% to 99.63%, respectively, whereas lineage 1 and 7 CoVs share nucleotide identities ranging from 71.07% to 72.59%. The lineage 7 *M. dasycneme* CoV sequences are most closely related to a CoV isolated from a *Rhinolophus* spp. sampled in the Shandong region in China (Tang et al. 2006), with identities ranging from 74.15% to 75.19%.

The bat-CoVs in lineages 1–4 and 7 are more related to hCoV-229E and hCoV-NL63 than to the CoVs isolated from *N. noctula* and the *P. pipistrellus* VM312 (nucleotide distance matrix available on request).

Discussion

A grounded *P. pipistrellus* bat in the Netherlands in 2008 was found positive for a group 2 CoV. The identification of *P. pipistrellus* as reservoir for group 2 CoVs is of importance for the assessment of the zoonotic risks of these viruses. *P. pipistrellus* is the most common and often most numerous bat species in the Netherlands and Western Europe. It has a preference for urban areas with roosting habits mainly in buildings (Kapteyn 1997, Dietz et al. 2009), bringing it in proximity to humans, thereby creating ample opportunities for (in)direct bat–human contact. *P. pipistrellus* is mainly a sedentary species and long-distance migration is rarely observed. In the Netherlands *P. pipistrellus* is the bat species with the most reported contact incidences with humans (Poel van der et al. 2005). In fact this particular specimen was found by humans in a residential quarter in a city.

The finding of group 2 CoVs in bats outside Asia supports the opinion that group 1 and 2 bat-CoVs will circulate worldwide, since recent evidence suggests that bat-CoVs are the gene pools for all the mammalian CoVs in groups 1 and 2 (Vijaykrishna et al. 2007, Woo et al. 2009). Whether this particular virus circulating in the Netherlands can cause zoonotic disease is not known. Further elucidation of its genome and corresponding characteristics is needed.

Our study provided new evidence supporting the observation that bat-CoV lineages might be associated with bat species rather than location. At location 9 both *M. daubentonii* and *M. dasycneme* CoV-positive bats were present. Each bat species was associated with a distinct CoV lineage that clustered with CoVs isolated from the same species but at different locations, even at large geographic distances as, for example, in Germany. No evidence for a common CoV cir-

culating between the two different bat species at location 9 was found. This reflects the local situation as both species originate from different roost habitats and location 9 is just a common foraging site for both species. This is indicative for a strict species association rather than a limited local transmission. Bat-CoVs associated with *N. noctula*, here identified as host for CoVs for the first time, captured at three different locations clustered together as well.

P. pipistrellus and *M. dasycneme* bats appeared to host phylogenetically distinct CoVs. The presence of both group 1 and 2 CoVs in one bat species has also been observed for *R. lechenaulti* in China (Woo et al. 2006) and *Hipposideros ruber* in Ghana (Pfefferle et al. 2009). In addition, *M. ricketti* and *R. ferrumequinum* bats were found to be associated with more than one CoV lineage (Tang et al. 2006, Woo et al. 2007). The closest relationship of the group 2 CoV sequence isolated from the Dutch *P. pipistrellus* was found with CoVs associated with Asian bats from the same genus, viz. *P. pipistrellus* and *P. abramus* (Tang et al. 2006, Woo et al. 2006). This close relationship of CoVs isolated from various species from the genus *Pipistrellus* was not observed within group 1 CoVs. The group 1 CoV associated with a Dutch *P. pipistrellus* clustered in a clade with the CoV sequences isolated from Dutch *N. noctula* bats and not with the German CoVs associated with the two other species in the genus *P. natushii* and *P. pygmeaus*. Molecular evidence suggests that the genus *Pipistrellus* is not monophyletic, and this might explain these differences (Jones et al. 2002).

The estimated prevalence of CoVs in bats in the Netherlands was 16.9%. This is 1.7 times higher than the prevalence determined in a German study sampling the same bat species but with a limited geographic distribution (Gloza-Rausch et al. 2008). Chinese studies sampling different bat species yielded prevalences ranging from 4.2% to 16% (Chu et al. 2006, Tang et al. 2006, Woo et al. 2006, Lau et al. 2007). Forty-eight percent of the sampled locations in the Netherlands were positive. Positive locations were found in western, eastern, and southern regions of the country, suggesting that CoVs circulate widely in bat populations in the Netherlands.

To improve our knowledge about CoV circulation in bat populations, we analyzed statistically the existence of predictive factors for CoV-positivity in bats. No predictive value of age, sex, ongoing lactation/gravidity, species, or sampling location for CoV detection was observed. In Germany, a positive correlation was found with juvenile age and ongoing lactation (Gloza-Rausch et al. 2008). A possible explanation for the observed discrepancy could be the limited sample size for some species and at some locations as reflected by the wide confidence interval.

Our results clearly support other observations that CoVs are diverse and widespread in bat species. Since there are >1100 bat species worldwide that account for 20% of all mammalian species, it is highly likely that the total extend of the diversity of CoVs in bats has not been addressed yet. Given the fact that (1) bats are considered as the natural hosts for all mammalian CoVs, (2) (bat-)CoVs have a propensity for interspecies jumping, and (3) certain bat species have habitats in proximity to human dwellings, one should be aware of the potential of bats as reservoirs for (highly) pathogenic zoonotic CoVs. The identification of a group 2 CoV in a bat species common to urban areas in most of Europe and notorious for its close contact with humans underlines the need for vigi-

lance. Therefore, the circulation of CoVs in bats should be monitored closely (diversity and prevalence) and its zoonotic potential should be investigated in more detail, including the influences of bat diversity and behavior.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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