1 **Title:**

2 **Novel Alphacoronaviruses and Paramyxoviruses co-circulate with Type 1 and**

3 **SARS-related Betacoronaviruses in synanthropic bats in Luxembourg.**

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14 Running Title: Viruses circulating among bats in Luxembourg

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ABSTRACT: 248 words

Several infectious disease outbreaks with high mortality in humans have been attributed to viruses that are thought to have evolved from bat viruses. In this study from Luxembourg, the genetic diversity and epidemiology of paramyxoviruses and coronaviruses shed by the bat species *Rhinolophus ferrumequinum* and *Myotis emarginatus* was evaluated. Faeces collection (n= 624) was performed longitudinally in a mixed-species colony in 2015 and 2016. In addition, faeces (n= 254) were collected cross-sectionally from six *Myotis emarginatus* colonies in 2016. Using degenerate primers in a nested format, an overall prevalence of 1.1% (10/878) and 4.9% (43/878) was determined for paramyxoviruses and coronaviruses. Sequences of the partial RNA-dependent RNA polymerase and spike glycoprotein genes of coronaviruses, as well as of the partial L-gene of paramyxoviruses were obtained. Novel paramyxovirus and *Alphacoronavirus* strains were identified in different *Myotis emarginatus* colonies, and SARS-related *Betacoronavirus* strains were shed by Rhinolophus ferrumequinum. Logistic regression revealed that shedding of *Alphacoronavirus* was highest in July (OR: 2.8, $p<0.01$), probably due to periparturient stress. Phylogenetic analyses point at close virus-host coevolution and the high genetic similarity of the study strains suggest that the *Myotis emarginatus* colonies in Luxembourg are socially connected. Most interestingly, we show that bats also host type 1 *Betacoronavirus* strains. The high similarity of the spike gene sequences of these viruses with mammalian type 1 *Betacoronavirus* strains may be of concern. Both the SARS-related and type 1 *Betacoronavirus* strains detected in bats in Luxembourg may cross the species barrier after a host adaptation process.

IMPORTANCE: 144 words

Bats are a natural reservoir of a number of zoonotic pathogens. Several severe outbreaks in humans (e.g. Nipah virus outbreak in Malaysia in 1998, and the almost global spread of Severe Acute Respiratory Syndrome in 2003) were caused by bat-borne viruses that were transmitted to humans mostly after virus adaptation (e.g. in intermediate animal hosts). Despite indigenousness of bat species that host viruses with suspected zoonotic potential and despite zoonotic transmission of European Bat Lyssavirus type 1 in Luxembourg, knowledge about the diversity and epidemiology of bat viruses remains limited in this country. Moreover, in contrast to other European countries, bat viruses are currently not included in the national surveillance activities of this land-locked country. We suggest that this gap in disease surveillance should be addressed as we show here that synanthropic bats host viruses that may be able to overcome the species barrier.

INTRODUCTION

Their ability to fly long distances and their longevity enable bats (Chiroptera) to spread viruses across time and space. Large colony sizes, close social interactions and co-roosting of different bat species favour intra- and interspecies transmission of viruses (1). Moreover and above all, low pathogenicity of viruses and viral persistence in bats are indicative of ancient co-speciation between bats and different virus families [e.g. Paramyxoviridae and Coronaviridae (2-5)]. It has been suggested that most human coronaviruses (CoV) evolved from bat counterparts (5-7). For instance Severe Acute Respiratory Syndrome (SARS) (8) and Middle East Respiratory Syndrome (MERS) CoV (9, 10), but also Nipah and Hendra paramyxoviruses (PV) (11, 12) originated in bats and caused severe outbreaks in humans. While for some viruses viral adaptation processes in intermediate animal

hosts were presumably required before zoonotic transmission (e.g. (9, 13)), direct transmission of Nipah virus between bats and humans repeatedly occurred in Bangladesh (14). The spike glycoprotein of several bat CoV strains share features with human strains which were critical for bat-to-human transmission events (15). In particular, the receptor-binding domain of the spike gene determines the host range and tissue tropism of CoV (16-18). Nevertheless, the risk of a zoonotic infection with bat viruses is low in humans as direct contacts with bat excretions are rare (19, 20). In addition, the risk can be monitored by virus surveillance in synanthropic bats (20), such as vespertilionid (e.g. *M. emarginatus*) and rhinolophid bats (e.g. *R. ferrumequinum*) that have been shown to host a number of viruses with zoonotic potential (5, 21-23).

In Western and Central Europe, *M. emarginatus* and *R. ferrumequinum* are endangered (48, 49) due to the on-going habitat fragmentation (24). After hibernating in underground sites, female *R. ferrumequinum* return in March to their natal colonies, while female *M. emarginatus* follow only in May (25-27). They form matrilineal maternity colonies in attics and barns (25-27). Around mid-June, females give birth to a single pup. Intra-lineage polygyny is common for *R. ferrumequinum* (28, 29) and extra-colony mating of *R. ferrumequinum* and *M. emarginatus* occurs during the swarming of the males, between September and October (30, 31).

Despite a growing interest in these animals as hosts of emerging viruses, the knowledge about bat viruses in Luxembourg remains limited. In a single study European Bat Lyssavirus type 1 was isolated and the risk of zoonotic transmission in the country shown (32).

Here, we report the shedding of PV and CoV by *R. ferrumequinum* and M. emarginatus, two sympatric and synanthropic bat species. Virus diversity and prevalence was assessed in six nursing colonies of *M. emarginatus* in a cross-sectional manner. In addition, we investigated seasonal patterns of both viruses in a mixed *R. ferrumequinum* / *M. emarginatus* colony, in a parallel longitudinal study. Several novel viruses of both families were detected and we show that bats are also a host for *BetaCoV* type 1.

MATERIALS

In 2015 and 2016, fecal samples (n= 624) were collected from a mixed *R. ferrumequinum* / *M. emarginatus* nursing colony in Bech-Kleinmacher, using a longitudinal approach. Samples were collected (i) after resettling of the colony in the summer roost and before birth of the juveniles (June 2015, n=100; May 2016, n=99), (ii) during lactation (July 2015, n=126; June 2016, n=111) and (iii) before the colony returned to the winter roost (September 2015, n=100; September 2016, n=88). In 2016, in the framework of a cross-sectional study, fecal samples (n= 254) were collected from 6 of the 14 synanthropic *M. emarginatus* colonies known in Luxembourg (Table 1, Figure 1). Beginning of June 2016 and before birth of the juveniles, the population size of every known *M. emarginatus* maternity colony in Luxembourg (Table 1, Figure 1A) was assessed by counting the bats emerging from the roost and/or the bats from a photograph taken in the roost, according to the Guidelines for Surveillance and Monitoring of European Bats (33).

The monitoring and sample collection was approved by the Ministry of Sustainable Development and Infrastructure Luxembourg (ref.: 86503 CG/ne).

Fresh faeces were collected on a clean tarpaulin (left for 2-12 hours underneath the roost) and individually placed into 2 ml tubes using single-use spatulas. Samples were kept at +4°C during transport to the laboratory, where they were directly processed. Bat species was identified by visual inspection of the faeces and of the bat cluster hanging above the collection site. Species identification was confirmed for virus-positive samples by sequencing of mitochondrial DNA (see below).

The study dataset is described in Table 1 and the primer sequences can be found in Table 2.

METHODS

Nucleic acid extraction. Entire bat droppings (approximately the size of a long grain of rice) were individually resuspended in 1 ml of prechilled virus transport medium (prepared according to the WHO protocol (34)) and homogenized using stainless steel beads (Qiagen, Venlo, The Netherlands) and a TissueLyser II (Qiagen).

After centrifugation at 2200 g for 20 min, the supernatant was transferred to a new 2 ml tube and stored at -80°C until further processing. Before nucleic acid extraction, each sample was centrifuged at 2200 g for 10 min and spiked with an extraction control (i.e. Human Adenovirus C5). Concurrent extraction of DNA and RNA was performed with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. To test for inhibition and confirm the successful extraction, each sample was tested using a real-time PCR specific for adenovirus (35).

Virus detection. All samples were tested for CoV and PV by reverse transcription PCRs with degenerate primers in a nested format. The PCRs were performed in a final volume of 25 µl. In the first step of the nested PCR, the QIAGEN One-Step RT-PCR Kit (Qiagen) was used. The mastermix of the CoV PCR contained 2µl of RNA,

136 1µM of each primer, 1mM of $MgCl_2$ and 1mM of each dNTP and the mastermix of
137 the PV PCR contained 250nM of each primer, 1.5mM of $MgCl_2$ and 100µM of each
138 dNTP. In the second step of the nested PCRs, the mastermix of the CoV PCR
139 contained 2.5 µl of 1:5 diluted PCR product, 700nM of each primer, 2mM of $MgCl_2$
140 and 200µM of each dNTP, whereas the mastermix of the PV PCR contained 0.1 µl of
141 undiluted PCR product, 600nM of each primer, 2mM of $MgCl_2$ and 200µM of each
142 dNTP. The adenovirus detection PCR was similar to the CoV PCR, but 2.5 µl of DNA
143 were used and 560nM of probe were added to the mix. In the second step of the
144 nested PCRs, in the adenovirus detection PCR and in the bat species identification
145 PCR, the Platinum® Taq DNA Polymerase Kit (Life Technologies Europe B.V., Gent,
146 Belgium) was used. The CoV primers target the RNA-dependent RNA polymerase
147 (RdRp) [modified from (36)], whereas the PV primers target the L-gene (37) of all
148 known strains of the respective viral families. An avian infectious bronchitis virus (an
149 avian CoV) and a measles virus (a human PV) served as positive controls in the CoV
150 and PV PCRs. Details about the primers can be found in Table 2.

151 **Sequencing**. PCR positive samples were identified by agarose gel-electrophoresis.
152 Where multiple bands were present, amplicons of the appropriate size were excised
153 from the gel and purified with the QIAquick gel extraction kit (Qiagen). PCR products
154 giving a single band in the gel-electrophoresis were directly purified using the
155 JetQuick™ extraction kit (Genomed, Löhne, Germany). Sequencing was performed
156 using the BigDye terminator kit (Applied Biosystems, Foster City, CA) run on an ABI
157 3130 sequencer (Applied Biosystems). Partial L-gene sequences of PV were
158 obtained using the detection primers. Partial sequencing of CoV was attempted
159 using specific primers targeting the conserved RdRp gene, as well as the spike
160 glycoprotein gene. To reliably identify the bat species of all virus-positive samples,

161 partial cytochrome b sequences were obtained. The bat species identification PCR
162 was performed using the Platinum® Taq DNA Polymerase Kit (Life Technologies
163 Europe B.V.) in a final volume of 25 µl containing 5 µl of DNA, 700nM of each
164 primer, 4mM of MgCl and 400µM of each dNTP. New primer sets were designed and
165 evaluated with Geneious software (version 7.1.7; Biomatters Limited; Auckland, New
166 Zealand [http://www.geneious.com]) and Primer3Plus
167 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Details about the
168 sequencing primers can be found in Table 2.

169 All viral and mitochondrial sequences were submitted to GenBank (accession
170 numbers KY502383 to KY502414, as well as KY707827 and MF048874 to
171 MF048903).

172 **Sequence and phylogenetic analyses.** Sequence assembling and processing was
173 performed in Geneious v.7.1.9 (http://www.geneious.com/; (38)). A BLASTn search
174 against the sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) was
175 performed with the default parameters. Phylogenetic trees based on nucleotide
176 sequences of the partial RdRp and spike genes for CoV, and of the partial L-gene for
177 PV were constructed. In order to increase the phylogenetic resolution and because
178 of the high genetic similarity of the obtained virus strains, only the longest sequences
179 of high quality were selected among the novel sequences, and aligned with
180 representative GenBank sequences using the ClustalW algorithm (39), as
181 implemented in Geneious. Poorly aligned positions in the alignments were eliminated
182 using Gblocks (40) as implemented in Seaview version 4 (41). Maximum likelihood
183 (ML) and Bayesian inference of evolution were estimated in PhyML (42, 43) and
184 BEAST (44, 45), respectively. The best substitution model identified by jModelTest
185 (46), according to the Bayesian Information Criterion (BIC) and/or Akaike Information

Criterion (AIC) values was used. A bootstrap test including 1000 replicates was performed for each ML tree. For the Bayesian Markov Chain Monte Carlo (MCMC) approach, the parametric model "Constant Size" was used as prior and the analyses were performed with a lognormal relaxed clock. The MCMC run was at least $2X10^7$ steps long with sampling every 10^3 steps. Convergence was assessed on the basis of the effective sampling size using Tracer version 1.6. (47). The results of the Bayesian phylogenetic inference were summarized in a maximum clade credibility tree using the Tree Annotator program after a 10% burn-in. Tree topology was tested by posterior probability (pp) and only the pp values of well supported nodes (pp>0.7) are shown. As the topologies of the trees based on Bayesian and ML inference largely overlapped, only the maximum clade credibility trees are shown. However, for the nodes also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in brackets. The scale bar of the trees indicates the average number of nucleotide substitutions per site (Figure 2-4).

Statistical analyses. Statistical analyses were performed in R software (version 3.1.0.; R Foundation for Statistical Computing, Vienna, Austria [https://www.r-project.org/]) (48). Logistic regression was performed to predict the binary outcome (i.e. presence or absence of detectable *AlphaCoV* shedding by M. emarginatus) based on the categorical predictor "season" with the levels "May/June", "July" and "September" and using a logistic function.

RESULTS

To assess the prevalence and diversity of PV and CoV shedding among bats in Luxembourg, fecal samples from 7 colonies (Figure 1A) were screened using degenerate primers in a nested format. The overall prevalence of PV was 1.1 % (10/878) and of CoV was 4.9% (43/878) and viruses were found in every colony

except for Colpach and Marienthal (Figure 1A, Table 1). No PV and CoV coinfections were detected.

Bat PV were only detected in Ettelbruck and Bech-Kleinmacher (Figure 1A), and shedding rates never exceeded 0.8-3.6% throughout the observation period. Because of the low prevalence rates, statistical analyses of the seasonal variation were not possible for PV. 9 out of the 10 detected PV strains were nearly identical to each other (represented by LUX15-A-033 and 351 in Figure 2). BLAST and phylogenetic analyses revealed that our PV strains were most closely related to those of insectivorous bats from China and South Africa with which they shared less than 80% nucleotide identity and less than 92% amino acid identity. Based on the phylogenetic analyses (Figure 2), all study sequences were grouped into a well-supported cluster, comprising also the unassigned murine J-virus (49), Beilong virus (50) and other Jeilongvirus-related PV (2, 3).

From the CoV strains detected in this study, partial RdRp gene sequences were obtained. We show that 2 out of the 4 currently recognized CoV genera (i.e. *AlphaCoV, BetaCoV, GammaCoV* and *DeltaCoV*) circulate in Luxembourg and 36 *AlphaCoV* and 7 *BetaCoV* were detected (Figure 3; Table 1). *M. emarginatus* from three different colonies (i.e. Ettelbruck, Lintgen and Bech-Kleinmacher; Figure 1A, Table 1) shed nearly identical *AlphaCoV* (>99 % nucleotide identity between partial RdRp gene sequences), most closely related to CoV circulating among insectivorous bats in China (Figure 3). In contrast to the PV shedding, *AlphaCoV* shedding was variable in Bech-Kleinmacher and highest rates were observed in July after parturition (OR: 2.8, $p<0.01$; Figure 1B). *AlphaCoV* strains from this study (represented by LUX15-A-48 in Figure 3) formed a distinct cluster and their RdRp

gene sequences shared less than 86% amino acid identity with other classified *AlphaCoV*.

On the phylogenetic tree of the partial RdRp gene, the *BetaCoV* strains from this study clustered within 2 out of the 4 recognized lineages (A to D) of *BetaCoV* (https://talk.ictvonline.org): *R. ferrumequinum* shed SARS-related CoV (lineage B, represented by LUX16-A-24 in Figure 3) and *M. emarginatus* shed *BetaCoV 1* (lineage A, represented by LUX15-A-158 in Figure 3). The SARS-related CoV from Bech-Kleinmacher were identical to each other and BLAST analyses revealed 94% nucleotide identity between partial RdRp gene sequences of this study and SARS-related CoV circulating among rhinolophid bats in Europe (Figure 1A, Figure 3). Besides, we detected the first bat *BetaCoV 1* strains (n= 5) in *M. emarginatus* from 3 different colonies in 2015 (Bech-Kleinmacher) and 2016 (Bissen and Platen) (Figure 1A, Table 1). All strains from Luxembourg were highly similar to each other and to *BetaCoV 1* identified in various mammalian species (>99% nucleotide identity between partial RdRp gene sequences) (Figures 3 and 4).

Sequencing of the partial spike gene was attempted for all novel bat CoV strains, but was successful only for the *BetaCoV 1* strains. As for the RdRp gene, all spike gene sequences were highly similar to each other and shared >98% nucleotide identity with the *BetaCoV 1* strains from other mammalian species (Figure 4B).

DISCUSSION

Bats are natural reservoirs of numerous viruses with zoonotic potential. Of particular interest are CoV and PV that share several traits allowing their adaptation to new ecological niches and hosts: high mutation rates, poor RNA proofreading capability and genetic recombinations (51-53). In line with previous studies [e.g. (3, 4, 23)], we

found genetically diverse CoV and PV strains in bats that are known to forage in and around human settlements in Luxembourg (Figures 2 to 4, Table 1). Shedding rates may have been under-estimated due to RNA degradation, as well as due to low viral loads in faeces (54) and reduced sensitivity of degenerate primers. However, the sample collection and processing protocol was optimized to minimize the degradation of viral particle and of RNA, as well as inhibition. We acknowledge that the adenovirus control did not control for inhibition during the reverse transcription step. Although somewhat more susceptible to PCR inhibition and RNA degradation, fecal samples have been systematically used before to investigate virus epidemiology and evolution (55-58). Moreover, faeces are collected non-invasively and are thus the preferred material when studying viruses circulating among these endangered species (59, 60).

Plowright et al proposed three scenarios to explain temporal variations in virus shedding in bats: (i) virus reactivation in persistently infected bats, (ii) seasonal epidemic cycles aligning with physiology of their life cycle, or (iii) transient epidemics due to waning immunity (19, 61). Similar to a previous study (4), we observed no temporal variation of PV shedding possibly because of the low prevalence of PV shedding. In contrast and comparable to another study (55), significant increase in shedding of *AlphaCoV* was found in July possibly due to the periparturient stress (55, 62) (Figure 1B).

The lack of similar reference sequences complicated the genetic and phylogenetic characterization of the detected virus strains. Nevertheless, we identified novel PV and *AlphaCoV* strains that are related to bat viruses from distant regions of the world (Figures 2 and 3). Also, according to the PV species discrimination criterion published before (i.e. amino acid distance above 7-7.5% in the L-gene) (4), the study

284 sequences may represent putative novel PV strains, but this finding needs
285 confirmation by whole genome sequencing. Based on the topologies of the
286 phylogenetic trees and BLASTn analyses, the new CoV obtained in this study were
287 found to be sufficiently divergent to represent a novel RGU based on amino acid
288 sequence analysis of the partial RdRp gene (5, 23). We found no evidence of inter-
289 species transmission, although a mixed-species colony was followed during 2 years
290 (Figure 1). Together these findings confirm previous studies suggesting an
291 association between *AlphaCoV* and host taxa rather than between geography and
292 viral evolution, and thus a close virus-host evolution (23, 63-65).

293 On the other hand, the detection of highly similar virus strains in different colonies
294 (Figure 1A, Table 1) is indicative of a social link between *M. emarginatus* colonies in
295 Luxembourg. This is of particular interest with respect to ongoing efforts for the
296 conservation of this species. Indeed, short foraging distances (24) and life-long roost
297 fidelity complicate the preservation of *M. emarginatus* (66, 67). Since migratory
298 distances of 35 to 126 km have been reported between summer and winter roosts
299 (66, 67), and since all Luxembourgish colonies are within 45 km of each other
300 (Figure 1A), bats from different colonies may assemble during autumn swarming of
301 the males (30, 68). Thus, male bats may play a particular role in virus transmission
302 which warrants further investigation. Thus, a better understanding of the dynamics of
303 bat-associated viruses may benefit these endangered species by indirectly providing
304 information about foraging and mating behaviour.

305 In contrast to *AlphaCoV*, host-switching is a major evolutionary mechanism of
306 BetaCoV. For instance, SARS- and MERS-CoV circulated in bats, before crossing
307 the species barrier to infect an intermediate host, which in turn infected humans (8,
308 13, 69, 70). Bat SARS-CoVs even use the same receptor for cell entry than their

309 human counterpart and they were detected in rhinolophid bats (8) that host also
310 genetically diverse SARS-related CoV (23, 71-74). Also in our study, *R.*
311 *ferrumequinum* from Bech-Kleinmacher shed SARS-related CoV strains (Figure 3).
312 Although it is unlikely that these CoV represent a direct threat for humans, the
313 potential risk of adaptation to the human host should not be ignored (75-77).
314 *BetaCoV 1* species is another exception to the typical host-specificity of CoV. The
315 species comprises highly similar viruses of distantly related mammals (6, 78-81) and
316 so far, only a single, short *BetaCoV 1* sequence was obtained from a bat (10). Most
317 interestingly, we show here that *M. emarginatus* from different roosts shed *BetaCoV*
318 *1* strains (Figure 1A) that are highly similar and closely related to *BetaCoV 1* strains
319 detected in various other amimal species (Figure 4). Most recent common ancestor
320 analyses of *BetaCoV 1* suggested that the group appeared only recently and has low
321 host-specificity (82-84). For example, *BetaCoV 1* strains detected in exotic ruminants
322 such as giraffes or antelopes are thought to represent spillover viruses of bovine
323 CoV that underwent adaptive mutations (78, 80). Moreover, a possible animal origin
324 of the human HCoV OC43 was revealed by molecular clock analysis of the spike
325 gene (83, 84) that provides indication about host range and tissue tropism. The
326 permissiveness of human cells to certain *BetaCoV 1* strains further underlines the
327 potential of *BetaCoV 1* to be transmitted across species (80, 82). Also here, all spike
328 gene sequences were highly similar to *BetaCoV 1* from other mammalian species
329 reflecting the genetic stability typical of the lineage (78, 85, 86). To further investigate
330 the role of bats as reservoir of *BetaCoV 1*, studies focusing on the host range of this
331 CoV species are warranted.

332 In conclusion, we showed that bats in Luxembourg, Western Europe, are hosts of
333 novel virus strains that may be able to overcome the species barrier. *BetaCoV 1*

strains with spike and RdRp genes genetically highly similar to mammalian strains were detected in synanthropic bats. In addition, we identified SARS-related CoV that may infect humans after a viral adaptation process (75-77). As shown before for bat lyssaviruses (32), our study highlights a certain risk for zoonotic transmission of bat viruses in particular since foraging and roosting sites of most indigenous bat species overlap with human and animal habitats. To mitigate this risk, it is important to monitor viruses circulating in synanthropic bats and putative intermediate hosts, and to identify factors that affect bat populations.

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611

612 TABLES AND FIGURE LEGENDS

613 Table 1 Characteristics of the different colonies and of the dataset, as well as detection rates of coronaviruses (CoV) and

614 paramyxoviruses (PV)

Location of colony (UTM coord.)	**Bat species**	**Population size on the 10/06/2016**	**Sample collection date**	**Sample size (% subset of population)**	**Viral nucleic acids detected in bat faeces**				
					all CoV* and ** (%)	***AlphaCoV** (%)*	***SARS-related CoV** (%)*	*BetaCoV 1** (%)*	**bat PV* (%)**
Lintgen (32U 293/5511)	*Myotis emarginatus*	65	10.06.2016	44 (67.7)	8 (18.2)	8 (18.2)	0 (0)	0 (0)	0 (0)
Ettelbruck (32U 291/5525)	*Myotis emarginatus*	220	10.06.2016	44 (20)	1 (2.3)	1 (2.3)	0 (0)	0 (0)	1 (2.3)
Marienthal (32U 288/5510)	*Myotis emarginatus*	45	03.06.2016	45 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bissen (32U 288/5519)	*Myotis emarginatus*	35	03.06.2016	33 (94.3)	1 (3)	0 (0)	0 (0)	1 (3)	0 (0)
Colpach (31U 703/5515)	*Myotis emarginatus*	70	10.06.2016	44 (62.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Platen (31U 711/5519)	*Myotis emarginatus*	60	16.06.2016	44 (73.3)	2 (4.6)	0 (0)	0 (0)	2 (4.6)	0 (0)
Bech-Kleinmacher (32U 308/5489)	*Myotis emarginatus and Rhinolophus ferrumequinum*	942	09.06.2015	100 (10.6)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)
			14.07.2015	126 (13.4)	15 (11.9)	15 (11.9)	0 (0)	0 (0)	1 (0.8)
			04.09.2015	100 (10.6)	7 (7)	6 (6)	0 (0)	1 (1)	2 (2)
			17.05.2016	99 (10.5)	3 (3)	1 (1)	2 (2)	0 (0)	1 (1)
			11.07.2016	111 (11.8)	5 (4.5)	5 (4.5)	0 (0)	0 (0)	4 (3.6)
			09.09.2016	88 (9.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total		**1437**		**878 (61.1)**	**43 (4.9)**	**36 (4.1)**	**2 (0.2)**	**5 (0.6)**	**10 (1.1)**

615 * detected in *Myotis emarginatus*, ** detected in *Rhinolophus ferrumequinus*, *** UTM coord.: Universal Transverse Mercator

616 coordinates

617

618 Table 2 Primers used for detection and sequencing of Coronaviruses and Paramyxoviruses

Virus	Target protein	Primer sense	Primer sequence (5`-3`)	Amplicon size (bp)	Reference
Detection					
Paramyxoviridae	L protein, subunit of RNA-dependent RNA polymerase	Forward	GAAGGITATTGTCAIAARNTNTGGAC	660	(37)
		Reverse	GCTGAAGTTACIGGITCICCDATRTTNC		
		Forward semi-nested PCR	GTTGCTTCAATGGTTCARGGNGAYAA	580	
Coronaviridae	Replicase polyprotein 1ab	Forward	GGKTGGGAYTAYCCKAARTG	602	(36)
		Reverse	TGYTGTSWRCARAAYTCRTG		
		Forward nested PCR	GGTTGGGACTATCCTAAGTGTGA	555	
		Reverse nested PCR	CCAACAYTTNGARTCWGCCAT		this study
Human adenovirus	Hexon gene	Forward	GCCACSGTGGGGTTYCTAAACTT	130	this study
		Reverse	GCCSCAGTGGKCDTACATGCACATC		this study
		Probe	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA		(35)
Sequencing					
Alphacoronavirus	Replicase polyprotein 1ab	Forward	TGTGAAGGCCTTACAGCGTC	670	this study
		Reverse	AGAGCCACAWACAACACACA		
	Replicase polyprotein 1ab	Forward	TGATGCAGCTGTYARAGACTTC	690	this study
		Reverse	CCAGAAGTCGTACCACCAGG		
Betacoronavirus 1	Replicase polyprotein 1ab	Forward	AGACATCGTCCCCATCCATC	729	this study
		Reverse	AGCTACACGTGGTGTTCCTG		
	Replicase polyprotein 1ab	Forward	CATATCATCCCAGCCGCCAT	584	this study
		Reverse	TGCTGTTTTAGTGTTGCGGC		
	Replicase polyprotein 1ab	Forward	CCGCTTGTTATAGCCGCAAC	613	this study
		Reverse	AGCGCTACTGAGTTTGCAGA		
	Spike gene	Forward	GTGAGCACTGTTCGGGTCTT	432	this study
		Reverse	AGCAATGCTGGTTCGGAAGA		
	Spike gene	Forward	ATGGCATTGGGATACAG	492	(80)
		Reverse	TAATGGAGAGGGCACCGACTT		
	Spike gene	Forward	GGGTTACACCTCTCACTTCT	767	(80)
		Reverse	GCAGGACAAGTGCCTATACC		
SARS-related Coronavirus	Replicase polyprotein 1ab	Forward	AGTTGAGGTGGTCGACAAGT	650	this study
		Reverse	GCAGTGGTAGCATCTCCTGA		
Bat species identification	Cytochrome b	Forward	ATGACCAACATTCGMAARTCYCAC	390	this study
		Reverse	TGATGACGGTTGCTCCTCA		

Figure 1 Municipalities with known *Myotis emarginatus* colonies in Luxembourg and circulation of coronavirus and paramyxovirus strains (A); Seasonality of Alphacoronavirus shedding in Bech-Kleinmacher (B).

(A) Sampled municipalities are in bold. The blue quadrant with the mixed colony of Bech-Kleinmacher as centre, has a radius of 45 km and embraces all investigated colonies. The base map is from the Land Registry Office of the Grand Duchy of Luxembourg. (B) Error bars represent the 95% confidence interval and * corresponds to $p<0.05$.

Figure 2 Phylogenetic analysis of the partial L gene of paramyxoviridae.

Bayesian analyses of a 410 nt long alignment comprising unique, partial L gene sequences of 34 paramyxovirus (PV) strains that represent all PV species recognized by the International Committee on Taxonomy of Viruses (ICTV), as well as unassigned, but putative novel PV species. Three out of the 10 PV from this study were added to the dataset to represent the genetic diversity of PV circulating in *Myotis emarginatus* in Luxembourg. Four Pneumoviridae strains served as outgroup for the phylogenetic analyses. The study sequences are in red and strains hosted by bats are highlighted in bold to show the high genetic diversity of bat PV. Only the pp values of well supported nodes (pp>0.7) are shown and if the nodes were also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in brackets. For each cluster, the PV species, as well as the virus family assignment are shown. The sequences were named, if the information was available, according to the following nomenclature: abbreviated virus name/host species/three-letter code of the country of origin/Genbank accession number. The following virus name abbreviations were used: PMPV-Pneumonia virus of mice;

643 HRSV-Human respiratory syncytial virus; HMPV-Human metapneumovirus; AMPV-
644 Avian metapneumovirus type; HPIV-Human parainfluenza virus; MapV-Mapuera
645 virus; LPMV- Porcine rubulavirus; SV-Simian virus; MuV-Mumps virus; TuV-Tuhoko
646 virus: TV-Tupaia paramyxovirus; MenV-Menangle virus; APMV-Avian
647 paramaxyovirus; MeV-Measles virus; PPRV-Peste des petits ruminants virus; DoV-
648 Dolphin morbillivirus; PDV-Phocine distemper virus; CDV-Canine distemper virus;
649 NaV-Nariva virus; MoV Mossman virus; BtV-Bat paramyxovirus; JV-J-virus; BV-
650 Beilong virus; NiV-Nipah virus; HeV-Hendra virus; BPIV-Bovine parainfluenza virus;
651 PPIV-Swine parainfluenza virus; HPIV-Human parainfluenza virus; SeV-Sendai
652 virus; AsaPV- Atlantic salmon paramyxovirus: AnV-Anaconda paramaxyovirus;
653 FDLV- Fer-de-Lance paramyxovirus.

654 **Figure 3 Phylogenetic analysis of the partial RNA-dependent RNA polymerase**
655 **gene of all Coronavirus genera**

656 Bayesian analyses of a 853 nt long alignment comprising unique, partial RNA-
657 dependent RNA polymerase gene sequences of 50 coronavirus (CoV) strains that
658 represent all CoV species recognized by the International Committee on Taxonomy
659 of Viruses (ICTV), as well as unassigned, but putative novel CoV species. Three out
660 of the 43 CoV from this study were added to the dataset to represent the genetic
661 diversity of CoV circulating in *Myotis emarginatus* and *Rhinolophus ferrumequinum*
662 in Luxembourg. The DeltaCoV strains served as outgroup for the phylogenetic
663 analyses. Only the pp values of well supported nodes (pp>0.7) are shown and if the
664 nodes were also supported by ML inference (bootstrap confidence levels above 0.7),
665 the bootstrap support is shown in brackets.

For each strain, the CoV genus assignment is shown. Assignment to recognized ICTV species is only shown for the study sequences that are highlighted in red. The country of origin of each strain is stressed in bold to show the vast geographic spread of CoV. The sequences were named, if the information was available, according to the following nomenclature: abbreviated virus name/virus strain/host species/three-letter code of the country of origin/year of sampling/specimen number_ Genbank accession number. The following virus name abbreviations were used: BtCoV-Bat coronavirus; PEDV-Porcine epidemic diarrhea virus; HCoV-Human coronavirus; TGEV- Transmissible gastroenteritis virus; PRCV-Porcine respiratory coronavirus; FCoV-Feline coronavirus; MiCoV-Mink coronavirus; CiCoV-Civet Severe acute respiratory syndrome CoV; RatCoV-Rat coronavirus; MHV-Murine hepatitis virus; PHEV-Porcine hemagglutinating encephalomyelitis virus; BCoV-Bovine coronavirus; CaCoV-Canine respiratory coronavirus; ECoV-Equine coronavirus; DrCoV- Dromedary camel coronavirus; WtDCoV-White-tailed deer coronavirus; GCoV-Giraffe coronavirus; AnCoV-Sable antelope coronavirus; WBkCoV-Waterbuck coronavirus; SdCoV-Sambar deer coronavirus; RabCoV-Rabbit coronavirus; HeCoV-Hedgehog coronavirus; IBV-Infectious bronchitis virus; CMCoV-Common moorhen coronavirus; WECoV-Wigeon coronavirus; BuCoV-Bulbul coronavirus; ThCoV-Thrush coronavirus; MuCoV-Munia coronavirus; PCoV-Porcine coronavirus; WiCoV-White-eye coronavirus; NHCoV-Night heron coronavirus.

Figure 4 Phylogenetic analysis of the partial RNA-dependent RNA polymerase gene (A) and partial spike glykoprotein gene (B) of Betacoronaviruses

Bayesian analyses of a 1771 nt long alignment comprising unique, partial RNA-dependent RNA polymerase gene sequences of 38 coronavirus (CoV) strains (A) and of a 911 nt long alignment comprising unique, partial spike glykoprotein gene

sequences of 34 CoV strains that represent all BetaCoV species recognized by the International Committee on Taxonomy of Viruses (ICTV). In addition, 1 out of the 5 highly similar BetaCoV 1 strains from this study was added to the dataset to show the genetic relationship of BetaCoV 1 circulating in Myotis emarginatus in Luxembourg to BetaCoV 1 of other host species. Only the pp values of well supported nodes (pp>0.7) are shown and if the nodes were also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in brackets. Assignment to recognized ICTV species is shown for each strain. The study sequence is highlighted in red and strains that were detected in bats are stressed in bold to show that most BetaCoV species comprise CoV strains that were initially detected in bats. The sequences were named, if the information was available, according to the following nomenclature: abbreviated virus name/virus strain/host species/three-letter code of the country of origin/year of sampling/specimen number_Genbank accession number. The following virus name abbreviations were used: BtCoV-Bat coronavirus; HCoV-Human coronavirus; CiCoV-Civet Severe acute respiratory syndrome CoV; RatCoV-Rat coronavirus; MHV-Murine hepatitis virus; PHEV-Porcine hemagglutinating encephalomyelitis virus; BCoV-Bovine coronavirus; CaCoV-Canine respiratory coronavirus; ECoV-Equine coronavirus; DrCoV- Dromedary camel coronavirus; WtDCoV-White-tailed deer coronavirus; GCoV-Giraffe coronavirus; AnCoV-Sable antelope coronavirus; WBkCoV-Waterbuck coronavirus; SdCoV-Sambar deer coronavirus; RabCoV-Rabbit coronavirus; HeCoV-Hedgehog coronavirus.