Common occurrence of Belerina virus, a novel paramyxovirus found in Belgian hedgehogs

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European hedgehogs (Erinaceus europaeus), also known as common hedgehogs, can be found throughout Western Europe, stretching from southern Italy to southern Scandinavia and including the Iberian Peninsula.¹ They can live in a variety of habitats and are often found in gardens and parks. In recent years, hedgehogs have gained popularity as household pets, especially in the Western world, although this typically concerns four-toed hedgehogs (Atelerix albiventris), as the keeping of European hedgehogs is illegal in most Western countries²–⁴. Furthermore, wild hedgehogs that are underweight or have been injured are often housed by people aiming to help them regain their strengths and are sometimes kept as pets once they have recovered.

Because of this close contact between humans and hedgehogs, some concerns have arisen in recent years about the potential of zoonotic diseases to be transferred from hedgehogs to humans⁴. European hedgehogs are known to carry a variety of bacterial (Salmonella, Yersinia Pseudotuberculosis ...) and fungal (Trichophyton, Microsporum) pathogens, and several cases of hedgehog-transferred zoonoses have been reported in the past decade⁵–⁹. Additionally, hedgehogs also harbor several zoonotic viruses, including rabies and tick-borne encephalitis virus, as well as other viruses of which the pathological and zoonotic potential remains to be fully elucidated¹⁰–¹³. An example of this is the finding of a paramyxovirus in European hedgehogs in 1981¹⁴. The family Paramyxoviridae is a family of single stranded, negative-sense RNA viruses that was recently divided into four subfamilies¹⁵. The largest of these subfamilies, the Orthoparamyxovirinae, is further subdivided into eight genera and comprises 34 recognized species¹⁶. In addition to fish and reptiles, members of this subfamily infect a wide variety of mammals¹⁷. Most orthoparamyxoviruses are known to cause disease in their respective hosts and some (Hendra virus, Nipah virus) are known zoonotic pathogens that can spread to humans¹⁸,¹⁹. Based on a neutralization assay using antisera against different paramyxoviruses, the aforementioned virus discovered by Vizoso and Thomas was thought to belong to the Morbillivirus group (now genus Morbillivirus, subfamily Orthoparamyxovirinae)¹⁴. The virus was isolated from the feces and lungs of a severely ill hedgehog and caused a symptomatology similar to Canine distemper virus, although it was also found in feces of multiple healthy animals. Unfortunately, the genome sequence of this virus was not determined.

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Fer-de-lance virus
Belerina virus
Mount mabu lophuromys paramyxovirus 2
Beilong virus
Pohorje myodes paramyxovirus 1
Genus \textit{Morbillivirus}
Genus \textit{Respirovirus}
Genus \textit{Henipavirus}
Genus \textit{Narmovirus}
Genus \textit{Aquaparamyxovirus}
Bat paramyxovirus (MG203877)
Bat paramyxovirus (KJ641657)
Miniopterus schreibersii paramyxovirus (KC154054)
Bat paramyxovirus (KJ641657)
Bat paramyxovirus (MG203878)
Bat paramyxovirus (MG203877)
Shaan virus (MG230624)
J-virus
Bat paramyxovirus (MG230624)
Here we report the complete genome sequence of Belerina virus, a putative new paramyxovirus species discovered in *Erinaceus europaeus*, which was detected in the context of a study aimed at virus discovery in different Belgian Eulipotyphla species. Belerina virus shows a relatively high prevalence in the hedgehogs screened in this study and appears to share similarities with paramyxoviruses found in bats.

**Results**

**Discovery of a novel paramyxovirus species.** We decided to use the Oxford Nanopore MinION to screen the total RNA, taken from the kidney of a European hedgehog, for the presence of previously undiscovered viruses. After running for 48 h, 5.1 M reads were produced, totaling 1.25 Gb after trimming. A tblastx search of all reads against the viral subset of the NCBI RefSeq database found 38 reads displaying limited similarity to different paramyxovirus genomes, indicating the presence of a novel paramyxovirus. Based on these 38 reads, we designed primer sets spanning the entirety of the viral genome and used these to determine the complete genome sequence of this virus, through a combination of PCR and 5′/3′ RACE, and subsequent Sanger sequencing. The complete genome (GenBank: MN561699) is 15,948 nucleotides long, thereby adhering to the so-called ‘rule-of-six’, which states that efficient replication of paramyxovirus genomes is dependent on their length being a multiple of six. This new virus was given the name ‘Belerina virus’, based on its location of origin (Belgium) and the identity of its host (*Erinaceus Europaeus*).

**Phylogetic analysis.** Bayesian-based phylogenetic analysis of all 34 currently recognized orthoparamyxovirus species, based on the amino acid sequence of the six major paramyxovirus ORFs (N, P/V/C, M, F, G and L), groups Belerina virus within the genus *Jeilongvirus* (Fig. 1a). This genus currently contains seven species, six rodent-borne paramyxoviruses and one paramyxovirus that was found in bat urine. Together with this bat-borne Shaan virus, Belerina virus seems to form a sister clade to the rodent-borne Jeilongviruses. Interestingly, if the tree is expanded with all available complete and near-complete (>90%) orthoparamyxovirus genomes, including several viruses isolated from different bat species, it becomes apparent that Belerina virus is phylogenetically most closely related to the bat paramyxovirus BtML-ParaV (KJ641657). Together with this bat virus, Belerina virus forms a sister clade to all other jeilongviruses, including the other bat viruses (Fig. 1b). As a consequence of the incorporation of partial genomes into the analysis, the alignment on which the tree is based needed to be trimmed at both ends, resulting in 3845 informative sites, compared to 4344 for the tree shown in Fig. 1a. However, as illustrated in Supplementary Fig. S1, the altered topology of the tree in Fig. 1b is independent of this alignment trimming and is caused by the addition of the partial genomes, highlighting the importance of their inclusion in the phylogenetic analysis.

**Genome organization of Belerina virus.** Members of the family *Paramyxoviridae* typically share a similar genome organization, characterized by six major ORFs. These ORFs encode the nucleocapsid (N), a phosphoprotein (P), a matrix protein (M), two membrane glycoproteins (F and G) and the viral polymerase (L). Through a combination of leaky scanning (C) and mRNA editing (V, W), some additional accessory proteins are usually expressed from the P ORF. The V, W RNA editing site is marked by a motif sequence (YTA AAA AAG GCA ), indicating that mRNA editing likely also occurs in the case of Belerina virus. The role of these two proteins (KJ641657), only one such ORF is present, encoding a transmembrane protein (‘TM’). In all other jeilongviruses, a second additional ORF is present, encoding a small hydrophobic protein (‘SH’). The role of these two proteins is not yet fully elucidated, although recent research has shown that for JV, the SH protein is involved in inhibiting the production of TNF-α, while the TM protein seems to stimulate cell-to-cell fusion. A SH protein is also present in a limited number of other paramyxoviruses (Avian avulavirus 6, Mammalian rubulavirus 5, Mojiang henipavirus, Mumps rubulavirus and Bat mumps rubulavirus), while the TM protein is found exclusively in members of the genus *Jeilongvirus*. The genome of Belerina virus encodes only the TM protein, although this protein is slightly larger (310 aa) than the TM proteins (218–275 aa) of other jeilongviruses.
Occurrence and spread in Belgium. We collected kidney tissue samples from 147 Belgian hedgehogs that were brought in to three different animal rescue shelters. In total, 57 animals tested positive for Belerina virus (38.78%). A map showing the origin of the different animals is shown in Fig. 2. Most samples originated from the east side of Flanders, where the largest Belgian animal rescue shelter is located (Natuurhulpcentrum vzw, Opglabbeek). Belerina virus was detected in animals from all three collection points, although a formal comparison of positivity rates is hindered by the disequilibrium in the amount of animals collected at the different sites.

Histological analysis. The animals collected during the last year of sample collection (10) were selected for later histological analysis. A detailed overview of the histological findings is shown in Supplementary Table S2. Two of these animals (animals 7 and 10) tested positive for Belerina virus and six animals were infected with lungworms (animals 2–7). The lungs of all animals had alveolar edema. The interstitial tissue of animal 10 showed only agonal lesions, while that of animal 7 displayed signs of pneumonia in the form of follicular hyperplasia and the presence of granulomas. However, this pneumonia is unlikely to be of viral origin, as two of the virus-negative animals (animals 2 and 4) displayed similar lesions. With the exception of the virus-negative animals 1 and 5 showing acute tubular necrosis, no kidney lesions were observed in any of the animals. Taken together, we observed no Belerina-virus specific pathology in the kidneys or lungs of the observed animals.

Discussion
In this study, we present the complete genome sequence of Belerina virus, a putative new paramyxovirus species found in European hedgehogs. Belerina virus clusters together with the bat-borne Shaan virus, forming a sister clade to the other members of the genus Jeilongvirus. Interestingly, Belerina virus does not encode an SH protein, like Shaan virus, but instead has the same unique genome structure as MMLPV-1/-2, containing seven major ORFs (N-P/V/C-M-F-TM-G-L). The phylogenetically basal position of these viruses, relative to other members of the genus jeilongvirus, seems to suggest that jeilongviruses have acquired their additional ORFs sequentially, starting with the acquisition of the TM ORF, followed later by the addition of the SH ORF. Additionally, this SH-ORF acquisition appears to have occurred twice, once in the rodent-borne jeilongvirus lineage and once in the case of Shaan virus. When the phylogenetic analysis is expanded to also include likely paramyxovirus species for which only partial, yet largely complete (> 90%), genomes are known: rodent paramyxovirus, feline paramyxovirus and four additional (partial) genomes of bat paramyxoviruses (Bat PV 16797, Bat PV 17770, BtMf-ParaV and BtMl-ParaV), Belerina virus now clusters with BtMl-ParaV, but separately from the other bat jeilongviruses. This clustering is also reflected in the genome organization of these viruses, with both Belerina virus and BtMl-ParaV lacking an SH ORF, while the four other bat virus genomes do presumably encode SH proteins, although both these SH proteins and these viruses’ TM proteins are significantly larger than the SH and TM proteins of other jeilongviruses (Fig. 3). Important to note is that the SH proteins of these four bat viruses and the SH proteins of other jeilongviruses share no significant sequence similarity. This seems to suggest that the acquisition of the SH ORF has occurred separately in two different lineages of jeilongviruses, assuming, as is the case for SH proteins of other paramyxoviruses, that the SH proteins of these different viruses are functional homologs.

Another interesting feature of jeilongviruses that sets them apart from other paramyxoviruses is the exceptional size of their attachment glycoprotein (‘G’). The paramyxovirus attachment glycoprotein, sometimes designated H(N) protein if the glycoprotein possesses hemagglutination (and neuraminidase) activity, is primarily involved in regulating the binding of virus particles to target cells. In all non-jeilongvirus paramyxoviruses, it has a length of ~ 600 amino acids, the majority of which form a large ectodomain that is present on the outside of the viral membrane. For jeilongviruses, however, the size of the G protein is highly variable and can reach...
lengths of up to ~1600 amino acids. Moreover, although these expansions of the G protein share no significant sequence similarity between different jeilongviruses, they do have a certain degree of structural homology, as they are all characterized by a high fraction of serine, threonine and proline. The G protein of Belerina virus, however, is only 597 amino acids long, and as such lacks this S/T/P-rich expansion of the ectodomain. Interestingly, the five bat jeilongviruses also lack this expansion of the G protein ectodomain, further illustrating the separate evolutionary history of the rodent-borne jeilongviruses. Based on these observations, it is clear that, even though members of the genus *Jeilongvirus* have several unique characteristics that set them apart from other paramyxoviruses, only the presence of a TM ORF is a characteristic shared by all jeilongviruses, while the occurrence of an SH ORF or the expansion of the G ORF seem to be limited to certain subclades. Based on the uniqueness of these subclades, it has previously been suggested that the genus *Jeilongvirus* could be split into separate genera, with Shaan virus representing a separate genus, tentatively called 'Shaanvirus'. However, even though there are clear differences between the different subclades, the genetic distance between all jeilongviruses is somewhat limited and seemingly insufficient to warrant the establishment of separate genera, especially when compared to the within-genus genetic distance of other genera.

For this study, 147 animals from different locations throughout Belgium were screened for the presence of Belerina virus. Almost 40% of these animals tested positive. It is possible that this number is an overestimation of the actual prevalence due to sampling bias. The animals tested in this study were all brought into animal rescue shelters because they were wounded, weakened, sick or dead. If Belerina virus actually causes disease in hedgehogs, this method of sample collection could result in an overestimation of virus prevalence in the Belgian hedgehog population. However, histological analysis of two virus-positive animals revealed no virus-specific kidney or lung lesions, hinting at a limited or sporadic pathogenicity (if any) of Belerina virus. Unfortunately, we were unable to acquire additional tissue samples for more extensive histological analyses. Until now, there has been only one report of paramyxoviruses causing disease in hedgehogs. In their report, Vizoso and Thomas describe the finding of a paramyxovirus in a hedgehog displaying symptoms akin to canine distemper. Based on neutralization assays, they concluded that the virus they found was most similar to viruses of the *Morbillivirus* group (now genus *Morbillivirus*, subfamily *Orthoparamyxovirinae*). It is also stated briefly that similar viruses could be isolated from apparently healthy hedgehogs. Based on the information given in their report, it is possible that the virus they described belongs to the same species as Belerina virus, but this could not be verified due to a lack of sequence information.

In summary, we report here the first description of Belerina virus, a novel paramyxovirus found in European hedgehogs. Belerina virus appears to have limited pathogenicity and seems to be prevalent in the Belgian
Bayesian phylogenetic trees. Markov chain Monte Carlo analyses were run until adequate effective sample size, using BEAST v1.10.4 as an adequate model to describe the amino acid substitution process. For the analysis of the genome of Belerina virus, we used the complete amino acid sequences of the N, P, M, F, G and L open reading frames (ORF) of all publicly available complete and near-complete orthoparamyxovirus genomes, aligned separately using MAFFT (v7.123b) and subsequently concatenated. The resulting alignment was trimmed using trimAL (v1.4.rev15) with the gappyout setting. Following selection of an LG + G + I model, using ProtTest (v3.4.2), as an adequate model to describe the amino acid substitution process, BEAST v1.10.4 was used to infer Bayesian phylogenetic trees. Markov chain Monte Carlo analyses were run until adequate effective sample size was achieved, and the resulting trees were visualized using FigTree (v1.4.3).
sizes (ESS > 200) were obtained. TreeAnnotator was used to summarize a maximum clade credibility tree from the posterior tree distribution, employing a burn-in of 10%. FigTree v1.4.3 was used to visualize the resulting tree.

**Data availability**
The complete Belerina virus genome sequence has been submitted to GenBank (accession number: MN561699).

Received: 23 July 2020; Accepted: 14 October 2020
Published online: 09 November 2020

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**Acknowledgements**
The authors wish to thank the people at the Natuurhulpcentrum vzw; Opplagbeek, the VOC Neteland; Herenthout, the VOC Merelbeke and the VOC Malderen for their assistance in the collection of animals. B.V. is supported by a FWO SB grant for strategic basic research of the “Fonds Wetenschappelijk Onderzoek”/Research Foundation Flanders (1S28617N).
Author contributions
B.V., V.V., and P.M. designed the study. B.V., V.V., M.M., E.V. and P.M. performed the experimental work. B.V. wrote the main manuscript text and B.V. and P.M. prepared the figures. All authors reviewed the manuscript and approved the final version.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-76419-1.

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