Isolation of infectious Lloviu virus from Schreiber’s bats in Hungary

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Some filoviruses can be transmitted to humans by zoonotic spillover events from their natural host and filovirus outbreaks have occurred with increasing frequency in the last years. The filovirus Lloviu virus (LLOV), was identified in 2002 in Schreiber’s bats (Miniopterus schreibersii) in Spain and was subsequently detected in bats in Hungary. Here we isolate infectious LLOV from the blood of a live sampled Schreiber’s bat in Hungary. The isolate is subsequently sequenced and cultured in the Miniopterus sp. kidney cell line SuBK12-08. It is furthermore able to infect monkey and human cells, suggesting that LLOV might have spillover potential. A multi-year surveillance of LLOV in bats in Hungary detects LLOV RNA in both deceased and live animals as well as in coupled ectoparasites from the families Nycteribiidae and Ixodidae. This correlates with LLOV seropositivity in sampled Schreiber’s bats. Our data support the role of bats, specifically Miniopterus schreibersii as hosts for LLOV in Europe. We suggest that bat-associated parasites might play a role in the natural ecology of filoviruses in temperate climate regions compared to filoviruses in the tropics.
Some members of the Filoviridae family are known to cause severe disease and frequent death in humans (e.g., ebolaviruses and marburgviruses), while there are other members with no known pathogenicity to humans (e.g., Reston virus). Among filoviruses, ebolaviruses have received the most attention, both by public health experts and the public, mainly due to the multiple documented human outbreaks, particularly the West African Ebola virus (EBOV) disease outbreak in 2013–2016 and the recent outbreaks in the Democratic Republic of the Congo and Guinea. The first direct evidence pointing towards bats as natural reservoirs for EBOV was published in 2005, when RNA and virus-specific antibodies were detected in three species of fruit bats captured in Gabon and the Democratic Republic of the Congo. To date, direct isolation of the infectious virus from a specific bat species has only been achieved in the case of Marburg virus (MARV), which leaves several open questions regarding the natural reservoir host for other filoviruses. During the past few years, increased scientific focus on these viruses has revealed the presence of several novel filoviruses in bats from Asia, Africa, and Europe. Currently, the only filovirus known to be endemic in Europe is Lloviu virus (LLOV), where genomic RNA was identified during the investigation of Schreiber’s bats (Miniopterus schreibersii) die-off events in the Iberian peninsula in 2002. After its initial discovery, no other reports were published with regards to the presence of this virus until 2016, when RT-PCR positive bat carcasses in Hungary confirmed a more widespread presence of LLOV in M. schreibersii across Europe. This was followed by a report on seropositivity among Schreiber’s bats in Spain, suggesting the circulation of LLOV in that area. The circumstances of the initial detection of LLOV in Hungary were similar to the original events in Spain, with multiple possibly related die-off events in the area, exclusively affecting Schreiber’s bat. Nearly two decades have now passed since the discovery of LLOV and many questions remain unresolved regarding the nature of the virus, the risk of zoonotic spillover, and most importantly, the pathogenic potential for bats and humans. Concerns regarding the possible pathogenicity of LLOV in humans remain high, since multiple studies have revealed its functional and genomic relatedness to EBOV. Recently, a recombinant LLOV (rLLOV) rescue system was developed, in which the missing LLOV genome termini were complemented by homologous EBOV or MARV sequences. Although rLLOV is able to infect known target cells of EBOV, including human primary macrophages, the inflammatory response in human macrophages, a hallmark of Ebola virus disease, is not induced by rLLOV. This suggests that LLOV might be able to infect humans, but the infection might not lead to disease, similar to the nonpathogenic Reston virus. Public health preparedness to filovirus zoonoses is highly related to the ecological attributes of wildlife hosts. Outbreaks in the human population are most likely initiated by spillover events from infected animals to humans. In the case of MARV, these spillover events have been reported to correlate with the life cycle of the bat host, with increased zoonotic events occurring during the birthing period. Importantly however, bat life cycles are different in temperate climate conditions than in the tropics. The annual life cycle of Schreiber’s bats has key differences from bats that live in the tropics, most importantly the period of hibernation.

In the past two decades, the identification of reservoir species of filoviruses has become a major focus of research, with numerous different vertebrate and arthropod taxa being studied. Interestingly, ectoparasites of bats have not been involved in many of these investigations, although they can carry several types of pathogens. Bat flies are the most common ectoparasites of bats. In the case of hippoboscid flies, both sexes feed on the blood of the host species. Members of the Nyceritiidae and Streblidae families are typically associated with specific host species and in general bat flies and bats show a strong coevolution. While bats are a major focus in viral emerging infectious disease research, the transmission patterns of these viruses within host bat populations and the role of their highly-specialized ectoparasites in this enzootic ecology have barely been investigated to date. Ectoparasites, however, represent perfect vector candidates for bat-to-bat transmission, including intraspecies and interspecies transmission. It is therefore important to understand the complex picture of LLOV transmission within bats and potential vector species, including ectoparasites, to get a clearer picture about the natural circulation of this, and other filoviruses.

In the present study, we sought to understand the biology of LLOV at a specific bat roost site in northeastern Hungary. We investigated the role of Schreiber’s bats in the natural circulation of LLOV and whether bat-associated parasites might play a role in the ecology of LLOV. We performed serologic, RT-PCR and sequencing-based surveillance on Schreiber’s bats from a site of prior LLOV detection in Hungary, following a step-by-step investigation strategy. A primary result was the isolation of infectious virus directly from a bat blood sample, which makes LLOV only the third member of the family Filoviridae ever isolated from bats and the first filovirus isolation of a non-Marburg or Ebola virus genus member.

We developed and used a deployable field-sequencing technology based on target enrichment method. It is not without precedent, as similar techniques were used extensively to sequence viruses from patient samples during the West African EBOV outbreak. Here, we publish the use of such technology directly on wildlife hosts, optimized for the Nanopore sequencing platform that is amenable for both field and laboratory use in future studies.

**Results**

**LLOV seropositivity in Schreiber’s bats.** Blood samples were collected from live bats and bat carcasses during the period of 2016–2020 at the site of LLOV detection in Northeastern Hungary in 2016. Live bats were apparently healthy, whilst dead bats were found in various conditions, possibly depending on the incubation period of the carcass within cave conditions. All details about the samples and animals, such as sex and collection date are listed as Supplementary Data 1. In order to perform neutralization tests to detect the presence of anti-LLOV antibodies, we created lentivirus particles pseudotyped with the LLOV or EBOV glycoprotein (GP). LLOV PVs did not cross react with EBOV convalescent serum (NIBSC WHO standard 15/262) (Supplementary Fig. 4). The converse neutralizing cross-reactivity was tested using bat sera and an EBOV PV target. However, due to the insufficient remaining volumes of bat sera only two LLOV nAb positive bat sera and two LLOV nAb negative bat sera could be tested, in addition to EBOV convalescent serum as a positive control. Importantly, no cross-reactivity was observed in these experiments (Supplementary Fig. 4). These findings are in accord with the lack of inter-genera filovirus cross-reactivity previously reported between Ebola and Marburg viruses using an indirect ELISA.

Following the cross-reactivity tests with EBOV, we used the LLOV PVs to perform antibody neutralization tests (PVNTs) on bat serum samples from both live and dead bats (Fig. 1, Table 1). Nine Schreiber’s bat carcasses were collected during the period between 2016 and 2019 and from these four tested seropositive for LLOV, with relatively high neutralization titres (254–3485) (Supplementary Data 1). We were also able to detect LLOV RNA in certain tissues of these carcasses with low copy numbers,
We also investigated serum samples taken from 74 live Schreiber’s bats from the first sampling event in September 2018. Seroprevalence among live-sampled bats was 9/74 (12.16%) with relatively weak to moderate titres (64–211 IC50), particularly compared to seropositive carcases (Fig. 1, Table 1).

| Animal ID | PVNT LLOV 1 IC50 | PVNT LLOV 2 IC50 | Mean IC50 | Serology | Real-time RT-PCR | Status | Collection date |
|-----------|------------------|------------------|-----------|----------|------------------|--------|-----------------|
| LLOV 98   | 211              | a                | 211       | Pos      | N/A              | Alive  | 2018.09.18.     |
| LLOV 99   | 156              | 174              | 165       | Pos      | N/A              | Alive  | 2018.09.18.     |
| LLOV 102  | 132              | a                | 132       | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 110  | 64               | a                | 64        | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 115  | 143              | 197              | 170       | Pos      | N/A              | Alive  | 2018.09.18.     |
| LLOV 118  | 155              | a                | 155       | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 130  | 177              | 59               | 118       | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 138  | 83               | a                | 83        | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 143  | 90               | a                | 90        | Pos      | Neg              | Alive  | 2018.09.18.     |
| LLOV 1c   | 768              | N/A              | 768       | Pos      | Pos (lung, spleen)| Dead   | 2016.02.11.     |
| LLOV 2c   | 2999             | 3972             | 3485      | Pos      | Neg              | Dead   | 2016.02.11.     |
| LLOV 169c | 172              | 337              | 254       | Pos      | Pos (lung, spleen)| Dead   | 2019.01.31.     |
| LLOV 170c | 757              | 1136             | 946       | Pos      | Neg              | Dead   | 2019.01.31.     |
| Control 1 | Non-neut.        | Non-neut.        | Neg       | Neg      | Neg              | Alive  | 2019.08.08.     |
| Control 2 | Non-neut.        | Non-neut.        | Neg       | Neg      | Neg              | Alive  | 2019.08.08.     |

*aPVNT not performed due to lack of sufficient serum for a repeat experiment.

#Borderline positive value.

*Dead at the original site of virus emergence, Northeast Hungary, Zemplén-Mt.; Control 1 (M. myotis) and Control 2 (M. schreibersi) were used as healthy negative control animals from a distant bat roost site in Southwestern Hungary.

detailed in Tables 1, 2. We also investigated serum samples taken from 74 live Schreiber’s bats from the first sampling event in September 2018. Seroprevalence among live-sampled bats was 9/74 (12.16%) with relatively weak to moderate titres (64–211 IC50), particularly compared to seropositive carcases (Fig. 1, Table 1). Unfortunately, the highly limited amount of blood samples from live animals precluded replicate testing in certain cases, also hindering the possibility of coupled serologic and RT-PCR
examination. Therefore we were able to conduct both serological and RT-PCR examination in only one case (LLOV_105) where LLOV RNA was detected along with seronegativity (Supplementary Data 1).

LLOV RNA in bats and related ectoparasites. After the verification of seropositivity in bats, we focused on detection of LLOV RNA via RT-PCR in consecutive sampling events to facilitate viral genome sequencing and virus isolation efforts. Regular checking for dead animals during the hibernation period resulted in 10 bat carcasses (nine Miniopterus schreibersii, one Myotis myotis). After dissection of these carcasses, we tested blood and multiple organ samples (brain, liver, lung, spleen, and kidney) for the presence of LLOV and detected viral RNA in two (2/5) dead bats from 2016 and one from 2018 (Table 2). Overall, LLOV prevalence among Miniopterus carcasses was 33.3% (3/9).

Notably, LLOV RNA was only detected in spleen and lung samples, with low genomic copy numbers in both organs comparing to live bat samples where we found one to three orders of magnitude higher amounts of viral genomic copies (Table 2).

Altogether, 779 samples (351 blood, 89 feces, 19 urine, 320 ectoparasites) were collected from live animals (351 Myotis myotis, 230—Miniopterus schreibersii, 2—Ixodes simplex, 4—Ixodes schlesi), 351 bats, the volume of these samples is routinely extremely small, and isolation experiments. Considering the small body size of these bats, the volume of these samples is routinely extremely small, usually between 5 and 20 µL. The initial in vitro isolation efforts, utilizing multiple cell lines (Vero E6—human kidney, Tbil1—blood cells) did not result in detectable LLOV replication. We neither observed any cytopathic effect in the cell culture experiments nor obtained positive real-time RT-PCR results after three blind passages.

After obtaining SuBK12-08 cells, we repeated these efforts using the only remaining LLOV_378 blood sample, since by this time we had used most of the positive sample aliquots in previous isolation efforts. SuBK12-08 is a Miniopterus schreibersii—originated, SV40 transformed kidney cell line. After the second blind passage, we observed a strong cytopathic effect (Fig. 3). Virus replication was further verified by real-time RT-PCR which resulted in lower real-time Ct value (18.469) than the original blood sample (30.1), clearly indicating an increase in viral genomic copy numbers. Viral titers of passage 4 virus were determined by tissue culture infectious dose (TCID50) assay on SuBK12-08 cells and were as high as 1.78 × 109 TCID50 units/ml. The established virus isolate was subjected for in vitro testing of other cell lines. Vero E6—human kidney and Tbil1—blood cells) did not result in detectable LLOV replication. We neither observed any cytopathic effect in cell culture experiments nor obtained positive real-time RT-PCR results after three blind passages.

Lloviu virus isolation on bat, monkey, and human cells. Freshly obtained blood samples from the animals previously denoted PCR positive (LLOV_329, LLOV_378) were used for in vitro isolation experiments. Considering the small body size of these bats, the volume of these samples is routinely extremely small, usually between 5 and 20 µL. The initial in vitro isolation efforts, utilizing multiple cell lines (Vero E6—human kidney, Tbil1—blood cells) did not result in detectable LLOV replication. We neither observed any cytopathic effect in cell culture experiments nor obtained positive real-time RT-PCR results after three blind passages.

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A detailed summary of all RT-PCR positive samples, including sample type/organ, CT values of RT-PCR reactions and indications of follow-up experiments are presented in Table 2.

Table 2 Detailed information about LLOV RNA-positive samples.

| Sample ID | Species               | Collection date | Tissue(s)            | Cycle threshold (Ct) | Genomic copy numbers (copies/mL) |
|-----------|-----------------------|----------------|----------------------|----------------------|----------------------------------|
| LLOV_1P   | Miniopterus schreibersi | 11.02.2016     | Lung; spleen         | 35.56; 35.30         | 1.4 × 10^6; 1.6 × 10^6          |
| LLOV_5P   | Miniopterus schreibersi | 11.02.2016     | Lung                 | 35.09                | 1.8 × 10^6                      |
| LLOV_105  | Miniopterus schreibersi | 18.09.2018     | Blood                | 26.16                | 2.3 × 10^8                      |
| LLOV_105_P | Nycteribia schmidlii (P) | 18.09.2018     | Whole specimen      | 34.08                | 3.2 × 10^6                      |
| LLOV_147  | Miniopterus schreibersi | 19.09.2018     | Blood                | 25.39                | 3.6 × 10^8                      |
| LLOV_147_P | Nycteribia schmidlii (P) | 19.09.2018     | Whole specimen      | 34.61                | 2.4 × 10^4                      |
| LLOV_169P | Miniopterus schreibersi | 31.01.2019     | Lung; spleen         | 38.59; 36.31         | 2.7 × 10^5; 9.4 × 10^5          |
| LLOV_329P | Miniopterus schreibersi | 23.09.2019     | Blood                | 30.27                | 2.5 × 10^7                      |
| LLOV_329_P1 | Ixodes simplex (P)  | 23.09.2019     | Whole specimen      | 36.10                | 1.1 × 10^6                      |
| LLOV_329_P2 | Nycteribia schmidlii (P) | 23.09.2019     | Whole specimen      | 35.78                | 1.3 × 10^6                      |
| LLOV_378P  | Miniopterus schreibersi | 24.09.2019     | Blood                | 30.10                | 2.8 × 10^7                      |
| LLOV_378_P1 | Nycteribia schmidlii (P) | 24.09.2019     | Whole specimen      | 34.53                | 2.5 × 10^6                      |

(P) ectoparasite.
(Carcasses.
Sample was used for in vitro isolation efforts.
Sample was subjected to viral genomic sequencing.
Sequencing of the viral genome
amplicon-based (https://artic.network/) method for sequencing
of replicating LLOV in the infected cells (Fig. 3).
the complete coding region of the LLOV genome32,36,37. Using
observed in mock-infected control cells, con
revealed a punctate pattern in LLOV-infected cells that was not
observed in mock-infected control cells, confirming the presence
of replicating LLOV in the infected cells (Fig. 3).

Sequencing of the viral genome. We developed an ARTIC-like
amplicon-based (https://artic.network/) method for sequencing
the complete coding region of the LLOV genome32,36,37. Using
this method, we were able to retrieve sequence data from the RT-
PCR positive bat from which virus was isolated (LLOV_378) and
a nearly complete genome from its associated bat fly ectoparasite
(LLOV_378_P1) (Table 2).

We generated sequence data for ~99% of the known LLOV
reference genome (NCBI accession NC_016144). In the case of
sample LLOV_378, 99.45% of the known genome was covered,
starting from reference nucleotide position 28 and ending at
18,853. The mean coverage of this sample using all the data which
was derived from the three different primer sets was 17,345×
(Supplementary Fig. 2). The consensus sequence derived from the
RT-PCR positive-associated parasite, sample LLOV_378_P1,
spanned positions 28 to 18,853, with one notable gap between
position 11,937 and 12,051. Importantly, there were no
differences between the bat and bat fly derived viral sequences.
Our LLOV sequence data are 99.198% identical to the reference
genome. We detected the highest level of sequence variation
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Discussion
Several novel members of the family Filoviridae have been dis-
covered in the last decade including some found in Europe and
Asia, raising the potential for the emergence of filoviruses outside
of Africa. There is a considerable knowledge gap regarding the
pathogenicity, animal hosts, and transmissibility of these newly
discovered viruses.

The most groundbreaking result presented here is the suc-
cessful isolation of infectious LLOV, from the blood sample of an
RT-PCR positive bat. The pronounced cytopathic effect of LLOV-
infected Miniopterus SuBK12-08 cells combined with the high
titers of virus stocks propagated in these cells suggest that
SuBK12-08 cells are highly permissive to LLOV infection. This,
along with our other results, provides the first indication for a
possible reservoir role of Schreiber’s bats, which warrants further
investigation to establish whether these bats can serve as
asymptomatic hosts for the LLOV in Europe. The isolation of the
virus opens the possibility for extended pathogenicity studies in
the near future. As we also present here, Lloviu virus has the
potential to infect human cells, therefore receptor identification,
gene expression and indeed antiviral studies are now a priority.

We describe the circulation of LLOV in a colony of Schreiber’s
bats over the span of several years. This study represents the first
detailed observation of the natural circulation of a filovirus in a
temperate climate region. In addition to virus isolation results, the
seropositivity and presence of LLOV RNA in these bats and the
continuous circulation of the virus are supportive for the putative
reservoir role of Schreiber’s bats as the natural reservoir hosts for
this virus38. However at this point we can only conclude these animals
as hosts for the virus in Europe. Our observations are partially in
line with the previous finding that Egyptian fruit bats (R.
aegyptiacus) are a reservoir for marburgviruses39,40. Other bat
species have been reported as hosts for other filoviruses, such as
the Bombali virus41. We detected LLOV RNA in multiple deceased bats, raising the possibility that these bats died as a

Fig. 2 Summary of tested bat samples, verified RT-PCR positive bats and their ectoparasites. a Number of LLOV-specific RT-PCR negative (lighter
color) and positive (darker color) samples in each category: blood or organs (blue), feces (brown), urine (yellow), ectoparasites (green). *During these
sampling events only passive monitoring was performed; (b) schematic representation of coupled bat-ectoparasite LLOV RT-PCR positivity.

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species have been reported as hosts for other filoviruses, such as
the Bombali virus41. We detected LLOV RNA in multiple deceased bats, raising the possibility that these bats died as a
consequence of infection. Interestingly, some individuals were found with respiratory tract bleeding, however there was no correlation to RT-PCR positivity (Supplementary Table). Notably, the viral load in dead animals were one to three order of magnitude lower than in the live animal samples. More sophisticated surveillance activities are necessary to better understand the nature of LLOV infection in bats and to clarify the possible role of the virus in the morbidity and mortality among these bats.

Although rare, the occurrence of both fatal and non-fatal outcomes of viral infections of bats is not unique. The best known pathogens to cause such patterns in bats are lyssaviruses\(^4\). We also developed a sensitive serological test based on pseudo-type virus neutralization that supports LLOV serosurveillance testing of bat sera samples. Before this study, only immunoblot analysis had been used for LLOV serologic examination in bats\(^1\). Our method enabled the serological study of live-sampled wild bats, showing a LLOV seroprevalence of 12.2% (9/74), which is lower than the previously published 36.5% seroprevalence from bats in Spain, though our test measures antibody neutralization rather than simply binding activity. This is also direct evidence of LLOV exposure in these bats, which supports the previous observation of a more extended spatio-temporal presence of the virus in Europe\(^1\).

To exclude possible cross-reactivity with different filovirus nAbs we performed cross-reactivity tests (Supplementary Fig. 4). Based on the numerous European studies, involving viral metagenomics or targeted surveillance there were no any indication for the presence of other filoviruses than LLOV within the colony. Based on these data we do not expect the presence of other filoviruses which may interfere with the neutralization experiments. Also, we used GP-based PVNA system which was previously described as the most specific antigen compared to NP and VP40 antigens of filoviruses\(^4\). Except for MARV in Egyptian fruit bats, there is little information about enzootic circulation of a filovirus among specific bat colony sites. MARV virus prevalence values seen in various countries range from 2.5% and 13.7%, with seasonal variability noted\(^6\). Based on previous studies, natural fluctuation of the age composition within a single colony and therefore the changing numbers of susceptible animals within the population may largely affect the prevalence\(^2\). In case of Pteropodidae (fruit bats), bi-annual birth pulses may facilitate the persistence of filoviruses\(^2\). Available data on MARV circulation highlighted juvenile bats (around ~6 months old) as particularly likely to be infected at specific times of the year. This seasonal emergence of juvenile Egyptian fruit bats coincides with increased risk of human infection\(^2\). In contrast to some tropical species, M. schreibersii bats have only one breeding season per year, and notably have a hibernation period. Considering these features, there should be other driving factors involved in LLOV circulation (Supplementary Fig. 1).

The hibernation may also alter the response to LLOV infection as reactivation of latent herpesvirus infection or prolonged
coronavirus infection were both reported in bats in previous studies. At this point it is hard to assess the causality of LLOV infection and fatal hibernation outcome in these bats, it is thus crucial to understand possible hibernation effects of LLOV infection to better understand the pathogenic potential of this virus to these bats.

We have also provided evidence of filovirus RNA presence in arthropods. However, the precise role of bat flies and other insect-arthropod vectors in the maintenance of the LLOV infection cycle is unknown. Further investigations are necessary to elucidate the role of these potential vectors in the transmission dynamics of LLOV. The identification of additional LLOV isolates from bats in different geographical locations and host species will be essential to understand the potential zoonotic risk associated with LLOV. Moreover, understanding the evolutionary relationships of LLOV with other filoviruses will provide insights into the potential pathogenic potential of this virus and its zoonotic potential.
ectoparasites in LLOV transmission is unknown. It is not yet clear whether these bat flies may be acting as natural vectors or mechanical vectors for LLOV transmission or are simply dead-end LLOV spillover hosts. A recent article reported the unsuccessful infection of bat flies with MARV, ruling out the biological vector competence but still considered the possibility of mechanical transmission. Our observations are in accordance with this recent report, since LLOV-positive ectoparasites were only retrieved from positive animals, suggesting the presence of the viral RNA in engorged parasites. Coupled sequencing results from host and parasite also support passive transmission without active viral replication, since no differences were found in the viral genomic sequences derived from the bat fly (LLOV_378_P1) and its bat host (LLOV_378). Given the current relatively limited dataset, it is not clear whether these ectoparasites play a role as a vector in the transmission of LLOV. However, we cannot rule out the possibility that these parasites serve as mechanical vectors in the transmission of LLOV, which warrants further studies. Compared to previous studies, Reston virus was not found to replicate in arthropod vectors and MARV RNA was not found in parasites on MARV-positive bats. Successful sequencing of LLOV RNA isolated from bat flies, resulting in high-quality sequence data, indirect LLOV surveillance could potentially be conducted by testing ectoparasites of M. schreibersii bats, obviating the need for invasive blood sampling of bats. This would provide the opportunity to conduct wide scale geographic surveillance to discover LLOV endemic regions and obtain a more detailed distribution map of LLOV infection among M. schreibersii populations. The reliability of ectoparasite-based surveillance strategy of bats was already reported for multiple bacterial species.

Based on our sequence data, we have shown insights into the natural genomic evolution of LLOV. Comparing the LLOV genomic sequence data from this study with the reference genome sequence from Spain, the level of LLOV sequence variation is consistent with other filovirus genera. For ebolaviruses and marburgviruses, the GP gene is a hotspot of nucleotide sequence variation. The consistency of elevated GP sequence variation compared to other genomic regions in all three genera (Ebola virus, Marburgvirus, and Cuevavirus) suggests similar genetic evolutionary driving forces on this region (e.g., immune evasion). Based on the presented data and considering the previous reports from Spain, there are multiple reports of LLOV infection in Schreiber’s bats, consistent with the idea that this species may serve as a natural reservoir of the virus, however, at this point we can only conclude the role of these animals as hosts for the virus in Europe. Despite many remaining questions, we provide evidence and further support for the role of bats in the natural circulation of filoviruses in general. The transmission cycle of LLOV may be complex and potentially different from filoviruses whose enzootic cycle occurs in tropical climates. As a major difference, we present data showing LLOV RNA-positive highly-specialized bat-associated ectoparasites, highlighting the importance of future research regarding the role of these insects in LLOV and other filovirus transmissions. Similar surveillance studies may greatly facilitate the discovery and understanding of novel filoviruses. Most importantly, we have isolated the infectious virus which represents the only filovirus that does not belong to the ebola- or marburg-virus genera, and it is the third successful isolation of a filovirus directly from bats, following MARV and RVAV virus, opening the door to further virological studies. Importantly, we show that multiple human cell lines are permissive to LLOV infection, which raises concerns about potential bat to human spillover events with LLOV in Europe and urges immediate pathogenicity and antiviral studies.

**Methods**

**Ethics and biosafety statement.** Bat sampling activities on a country-wide scale were approved by the Hungarian Government Office of Pest County under the registration number of PE-KTFO/4384-24/2018. Animal handling was performed by licensed chiropterologists, no animals were harmed during the study, and all ethical standards were followed during the work. All in vitro virus isolation procedures were performed under Biosafety Level 4 conditions within the laboratory of Szentágothai Research Center, University of Pécs, Hungary.

**Animal sampling.** We conducted regular bat colony monitoring during the hibernation period (between November and March) (passive surveillance) at the site of LLOV re-emergence in Hungary (Northeast Hungary, Zemplén Mountains) from 2016 to 2020, collecting only deceased bats, if found. Whole carcasses were frozen and transferred in a dry shipper to the biosafety level 4 (BSL-4) laboratory at Szentágothai Research Center, Pécs, Hungary, where they were dissected. Lung, spleen, kidney, liver, and brain tissues as well as serum samples were stored at −80 °C until further analyses.

In addition, seven live animal sampling events (active surveillance) were performed between 2018 and 2020, when samples (blood, urine, feces, and ectoparasites) were collected from Schreiber’s bats (Miniopterus schreibersii) and Greater mouse-eared bats (Myotis myotis). Bat species identification was performed by trained chiropterologists according to morphological identification keys.

**Virus detection.** For LLOV RNA detection, we performed RT-PCR tests from all sample types (lung, spleen, kidney, liver, brain tissues, and blood from carcasses; blood, urine, feces samples and ectoparasite specimens from live bats). For blood samples in VTM, we carried out on-site LLOV RNA detection in the field, while other samples were transported to the laboratory for testing. These blood samples were further screened with a pan-filovirus nested-RT-PCR, previously used to detect several novel filoviruses in China.

**Tissues, feces, and ectoparasites** were homogenized in 100 μL 1x Phosphate Buffered Saline (PBS) using Minilys Personal Homogenizer (Bertin, Germany) with glass beads. The urine samples were complemented with 1x PBS to a total volume of 100 μL. Then, the tissues were centrifuged at 16,000 g for 5 min and 100 μL supernatant was transferred for RNA extraction. The 100 μL blood samples in VTM were directly used for RNA extraction.

RNA was extracted using Direct-Zol RNA MiniPrep (Zymo Research, USA). RNA was then used for RT-PCR using the QuIAGEN OneStep RT-PCR kit (Qiagen, Germany) at 50 °C for 30 min, and 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 40 s (the fluorescence signal was detected during the annealing step). We used the primers FiloAneu 5'-AGT CMT TYC CAN GYA AYA TGA TGG T-3' and FiloBNeo 5'-RTG WGG NRG RYT RTA AWA RTC ACT NAC ATG-3' and the probe LlovuS: S-FAM-5’-CTA AGA TTT CCG TGT TCA TGA TCC CA-BHQ1-3’ (it is noteworthy that a previously described detection method published by our laboratory resulted in a significant number of false positives, which in certain cases we subsequently failed to validate with a pan-filovirus nested-PCR method and sequencing). All experiments were run on the
MyGo Pro PCR system platform (IT-LS Life Science, Ireland) and analysed on the MyGo PCR software (v.3.5.21).

**Virus isolation.** Cell lines used in this study: Cercopithecus aethiops (African green monkey) kidney (Vero E6; ATCC CRL-1586), Mason’s guereza cells (SusBK12-08, kindly provided by Ayato Takada, Hokkaido University)18,35, human neuroblastoma cells (SH-SYSY; ATCC® CR-2266), human hepatocellular carcinoma (Hep G2; ATCC HB-8065®), human non-small cell lung carcinoma (HCC-78; DSMZ ACC 563), human colon carcinoma (HCT-116; DSMZ ACC 581), Tadarida brasiliensis lung cells (Tb 1 Lu; ATCC® CCL-88®).

VeroE6 cells were maintained in Dulbecco’s modified Eagle medium (DMEM, Lonza Cat. No: 12-604 F), SH-SYSY and Hep G2 cells were maintained in DMEM with 1% of MEM Non-essential Amino Acid Solution 100x (NEAA) (Sigma Cat. No: M7145), SuBK12-08 and Tb 1 Lu in Eagle’s Minimum Essential Medium (MEM, Lonza Cat. No: 12-991 F) with 1% of L-Glutamine (200 mM) (Lonza Cat. No: BE17-605E), HCC-78 in RPMI-1640 (Sigma Cat. No: R8186), McCoy’s 5 A (Lonza, Cat. No: BE12-688F). All culture medium was supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco Cat. No: 16140071), 1% of MEM Non-essential Amino Acid Solution 100x (NEAA) (Sigma Cat. No: M7145), 1% of L-Glutamine (200 mM) (Lonza Cat. No: BE17-605E), McCoy’s 5 A (Lonza, Cat. No: BE12-688F). All culture medium was supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco Cat. No: 16140071), 1% of MEM Non-essential Amino Acid Solution 100x (NEAA) (Sigma Cat. No: M7145), 1% of L-Glutamine (200 mM) (Lonza Cat. No: BE17-605E), HCC-78 in RPMI-1640 (Sigma Cat. No: R8186), McCoy’s 5 A (Lonza, Cat. No: BE12-688F).

FRESHLY OBTAINED blood samples from field PCR-tested LLOV RNA-positive bats were frozen in liquid nitrogen and transferred to the BSL-4 laboratory at the Szentágothai Research Center to perform virus isolation experiments. Of note, due to the low body weight of the animals, the blood sample volumes are extremely low, usually below 50 μL. The blood samples (5–50 μL, depending on the original amount) were complemented with cell culture media to a volume of up to 50 μL and used to inoculate monolayers seeded in 24-well plates with ~80% confluency. After 1 h incubation at 37°C and 5% CO2, the cells were washed once with cell culture media. The cells were monitored daily for cytopathic effects. After 7 days, both freeze-thaw cycles before culture supernatants were transferred to fresh cells. After three blind cycles by the freeze-thaw method, subjected to nucleic acid extraction by Direct-Zol RNA MiniPrep (Zymo Research, USA) and analyzed for the presence of LLOV RNA by RT-PCR using LLOV-specific primers. Viral titers were determined by tissue culture infectious dose (TCID50) assays on SuBK12-08 cells using the Spearman-Karber algorithm.

**RNA fluorescence in situ hybridization analysis.** For RNA fluorescent in situ hybridization (FISH) analysis, Vero E6 cells seeded in 8-well chamber slides were mock-infected or infected with LLOV at an MOI of 1. Cells were fixed 1 day post-infection in 10% formalin for at least 6 h. RNA FISH was performed using the RNAseq Multiplex Fluorescent V2 kit (Advanced Cell Diagnostics). Viral RNA was detected using custom-designed probes targeting the negative-sense genomic sequence of the LLOV NP gene (Advanced Cell Diagnostics) and stained with Olyp 690 fluorophore (Perkin-Elmer). Staining was performed according to the manufacturer’s protocol for adherent cell samples, with the exception of an additional HRP blocking step following signal development of the probes detecting viral mRNA as per the manufacturer’s recommendation. Nuclei were stained with kit-supplied DAPI. The slides were dried and mounted on slides using FluorSave mounting medium, and slides were subsequently stored at 4°C prior to imaging. Images were acquired at xx0 magnification using a Nikon Ti2 Eclipse microscope and Photometrics Prime BSI camera with NIS Elements AR software.

**Generation of LLOV pseudotyped lentiviruses and neutralization tests.** The LLOV GP gene (GenBank accession JF828358) was inserted into the pCAGGS expression plasmid (kind gift of Prof Ayato Takada). Generation of LLOV and EBOV GP pseudotyped lentiviruses was based on a protocol described previously69, with co-transfection of three plasmids, pCAGGS-LLOV GP or pCAGGS-EBOV GP, p8.91 (HV-1 gag-pol) and pCSFLW (luciferase reporter), into HEK293/17 cells, using 1 mg/mL branched Polyethyleneimine (PEI) (Sigma-Aldrich, USA) at a ratio of 1:100 (μg DNA: μL PEI). Pseudotyped lentivirus (PV) supernatant was harvested 48 h later and stored at ~80°C. Titration of PVs was carried out as described elsewhere90 by twofold serial dilution across white Nunc flat-bottomed 96-well microplates (Thermo Fisher Scientific, USA) in HEK293/17 target cells (2 x 104/well), incubated at 37°C, 5% CO2. After 48 h, the media was removed and discarded. Bright Glo reagent was added to the plate and incubated at room temperature for 5 min before measuring luminescence on a GloMax 96 luminometer (Promega, USA), with titers given in Relative Light Units (RLU) per mL. For ease of inter-lab comparison, TCID50 titers were also obtained by the same methodology but using a fivefold dilution series.

Luminescence values were used to calculate the PV titre (TCID50/mL) using the Reed-Muench method91. The cumulative number of positive and negative wells for 10% neutralization) or when a neutralization of <70% was achieved. The mean of these values was calculated and a cut-off was defined for values higher than the mean + three times the standard deviation63-65 (Supplementary Fig. 5).

**Amplicon-based nanopore sequencing.** We developed an amplicon sequencing method based on previous protocols66,67 that is able to amplify the LLOV-specific gene regions in two parallel multiplex PCR reactions29. LLOV RNA-positive samples LLOV-378 and LLOV-378F1 were used for sequencing. cDNA preparation from RNA samples was conducted with SuperScript IV (Invitrogen, USA) with random hexamers. Amplicons were generated from cDNA with Q5 Hot Start HF Polymerase (New England Biolabs, USA), with three different primer sets in parallel pools (namely: LLOV_400bp pool 1 and 2, LLOV_500bp pool 1 and 2, LLOV_2000bp pool 1 and 2) (Supplementary Fig. 2). 2.5 μL amplicons from each pool were sent to the same-primed sequencing. Pooled amplicons were ligated with NEBNext Ultra II Ligation Module (New England Biolabs, USA). 1.5 μL end prepped DNA was transferred to the next reaction directly and the ligation performed with EXP-NBD196 (Oxford Nanopore Technologies, UK).

After the clean up of the pooled different barcoded samples jointly with Ampure XP beads (Beckman Coulter, USA), the AMⅡ sequencing adapters were ligated with NEBNext Quick Ligation Module. The final library was quantified with Qubit dsDNA HS Assay Kit (Invitrogen, USA) on a Qubit 3 fluorometer. 25 ng of each library was pooled and loaded on a R9.4.1 (FLU-MIN106D) flow cell and were sequenced for 48 h97.

**Bioinformatic pipeline.** The raw sequencing data werebase called using guppy (ONT guppy v4.4.2) high accuracy basecaller algorithm (dna_r9.1.4,50bps,bac config file). Demultiplexing and trimming of barcodes were performed also with guppy using default parameters of guppy_barcode’ runcode. The ONT guppy software was used under Ubuntu Linux 18.04. Because ARTIC-like protocols generate chimeric reads, we performed length filtering with appropriate lengths specific to the used primer set (LLOV_400bp: 350–600 bp, LLOV_500bp: 400–700 bp, LLOV_2000bp: 1400–2700 bp). Primers were trimmed with the BBDuk (v38.84) Geneious Prime (v2021.1.1) plugin. To generate a consensus sequence, the processed reads from all samples were mapped to the LLOV reference genome (NCBI accession number: NC_016144) with the usage of Minimap 2.1.796. The generated consensus sequences were manually checked for basecalling errors.

**Sequencing of the virus isolate.** Prior to Nanopore sequencing, virus isolates were exposed to a generally used enrichment protocol66,70. Nucleic acid was extracted from samples using the Direct-zol RNA Miniprep Plus Kit (Zymo Research, USA). Samples were then subjected to the Sequence Independent Single Primer Amplification (SISPA) approach with minor modifications to the previously described protocol69. cDNA amplification was performed by SuperScript IV Reverse Transcriptase (Thermo Fisher scientific) with dNTPs (10 μM) and 2 μM K-8N primer following the manufacturer’s instructions. In order to convert double stranded cDNA from first strand cDNA, samples were subjected to Klenow reac- tion. cDNA was end prepped with NEBNext End Prepping Module (New England Biolabs, USA) and were barcoded with EXP-NBD196 (Oxford Nanopore Technologies, UK) kit. The final library was sequenced on a R9.4.1 (FLU-MIN106D) flow cell.

The raw sequencing data were base called using guppy (ONT guppy v5.0.7) super-accuracy basecaller algorithm (dna_r9.1.4,50bps, sup config file). Demultiplexing and trimming of barcodes were performed also with guppy using default parameters of guppy_barcode’ runcode. The ONT guppy software was used under Ubuntu Linux 18.04. Porch v0.2.4 was used to trim primer sequences used for SISPA amplification. To generate a consensus sequence, Medaka v1.4.2 was used to map the trimmed reads against the LLOV reference genome (NCBI accession number: NC_016144) and perform variant calling. Extension of the genome ends was done manually filling in the observed
overhangs based on a mapping performed using the Long Read Support tool in CLC Genomics workbench v20.0.4. Extension was stopped when read coverage fell below 20x.

**Phylogenetic tree capture.** Prior to the phylogenetic tree implementation, 22 full length sequences belonging to the family Filoviridae were aligned in the MAFFT webserver (version 7 [https://mafft.cbrc.jp/alignment/server/]) using default parameters. Subsequently, a best substitution model selection was performed in IQ-TREE webserver ([http://iqtree.cibiv.universite-udine.it](http://iqtree.cibiv.universite-udine.it)) and thus a Bayesian phylogeny tree was generated using BEAST v1.10.4 [1] under a GTR + G + I substitution model, assuming a constant population size and a strict molecular clock (uniform rates across branches). The MCMC chains were run for 10,000,000 iterations and sampled each 1,000th generation. The effective sampling size was checked in Tracer software (>200). Thereafter, the Maximum clade credibility tree (MCC) was generated using TreeAnnotator and edited in the iTol webserver ([https://itol.embl.de/]).

**Statistics and reproducibility.** Depending on the available amount of sera different measurement and dilution strategies were applied: measured as duplicate with repeated experiment—bat $f_1$, $f_16$, $f_{170}$, $f_{299}$, $f_{115}$, $f_{130}$; measured in duplicate with single experiment—bat $f_2$, $f_{102}$, $f_{138}$; measured in single in one experiment—bat $f_{298}$, $f_{110}$, $f_{118}$, $f_{143}$. All dilutions, including standard sera were 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120 except for bat $f_{143}$ where 1/100 starting dilution was applied.

In vitro isolation experiments were performed in triplicates with same results. The RNA FISH analysis was performed once. The probes were evaluated with 100 starting dilution was applied.

**Results and Discussion**

- The genomic sequence data in this study have been deposited in the NCBI GenBank database under accession codes: MW775010, MW775011 and MZ541881. Tick samples: OL799929-OL799963 ([https://www.ncbi.nlm.nih.gov/nuccore/?term=olovin+hungary](https://www.ncbi.nlm.nih.gov/nuccore/?term=olovin+hungary)). The background data of sampled animals, such as sex and collection dates are provided in the Supplementary Data 1. Sequencing protocol and materials are listed here: [https://www.protocols.io/view/llovirus-cuevavirus-sequencing-protocol-bmz3k78n.html](https://www.protocols.io/view/llovirus-cuevavirus-sequencing-protocol-bmz3k78n.html).

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**Conflict of interest**

The authors declare no conflicts of interest.
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Author contributions

G.K., G.E.T., M.M.N., S.S., B.Z., Z.L., Á.N., C.I.P., G.D., F.F. conceived the laboratory work. G.K., G.E.T., S.A.B., T.G., P.E., Z.L., Á.N., C.I.P., G.D., K.K. conducted field work. M.M.N., S.S., N.T., E.W. designed and conducted the neutralization experiments. S.A.B., T.G., P.E. handling of bats during field work. P.E., T.S. ectoparasite identification. G.K., M.M., F.F. conceived the in vitro isolation experiments. E.L.S. performed the RNA FISH experiments. G.E.T., B.A.S., A.J.H., S.Z., G.K., P.M., B.V. sequencing and genomic analysis. B.A.S., M.M.N. visualization of results. G.K., F.J., S.S. conceptualization and supervision. G.K. wrote the paper. G.E.T., M.M.N., S.S., N.T., E.W., S.A.B., T.G., A.N., F.I., A.J.H., E.M. edited the paper with contributions from all other authors. G.K., S.S., E.M., Á.N., F.J. provided supervision. All authors approved the paper.

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Competing interests

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Additional information

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