Fecal virome analysis of three carnivores reveals a novel nodavirus and multiple gemycircularviruses

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Abstract

Background: More knowledge about viral populations in wild animals is needed in order to better understand and assess the risk of zoonotic diseases. In this study we performed viral metagenomic analysis of fecal samples from three healthy carnivores: a badger (Meles meles), a mongoose (Herpestes ichneumon) and an otter (Lutra lutra) from Portugal.

Results: We detected the presence of novel highly divergent viruses in the fecal material of the carnivores analyzed, such as five gemycircularviruses. Four of these gemycircularviruses were found in the mongoose and one in the badger. In addition we also identified an RNA-dependent RNA polymerase gene from a putative novel member of the Nodaviridae family in the fecal material of the otter.

Conclusions: Together these results underline that many novel viruses are yet to be discovered and that fecal associated viruses are not always related to disease. Our study expands the knowledge of viral species present in the gut, although the interpretation of the true host species of such novel viruses needs to be reviewed with great caution.

Keywords: Virome, Gemycircularvirus, Metagenomics, viral discovery

Background

With the advent of next generation sequencing techniques, samples from a wide range of animal species have been screened to identify novel viruses and this approach has become the most important tool for early detection and characterization of possible emerging zoonotic agents [1–3]. It is important to monitor these emerging zoonotic agents as they can be responsible for minor or major epidemics worldwide [4]. For example, zoonoses can range from the Middle East respiratory syndrome coronavirus (MERS-CoV), which recently drew a lot of attention worldwide [5], to the 2009 pandemic H1N1 influenza A virus or to the less publicized Hepatitis E virus [6]. Not only are humans at risk, but also animals can be infected with viruses from other host species, resulting in disease, or further transmission to humans. The severe acute respiratory syndrome (SARS) coronavirus pandemic originated from wildlife, where bats where identified as the reservoir and civets as an intermediate host [7, 8]. A similar example comes from Hendra virus, which also originated from bats, but transmission to humans occurred with horses as an intermediate host, causing severe pneumonia in horses and humans [9]. As such, zoonotic infections can have significant consequences for animal and public welfare. However, little is known about these pathogens before they emerge from unrecognized zoonotic sources and therefore a deeper understanding of the virome of wild animals will allow us to more rapidly identify the host of particular novel zoonotic viruses, and act appropriately to prevent further spread of such viruses.

Fourteen species of wild carnivores can be found in Portugal, often in relative close contact with humans. Only red fox, stone marten, badger, common genet and, more recently, the Egyptian mongoose, are known to have a generalized distribution throughout the country [10]. From these animals we sampled two species, a badger (Meles meles) and an Egyptian mongoose...
Geminiviridae family comprises two genera, *Mycelium parvum* and *Mycelium geminigerum*. *Mycelium parvum* is restricted to plants and not known to infect animals. *Mycelium geminigerum* shares 71.7% amino-acid similarity with *Mycelium parvum*, raised in a zoo, since otters and *Mycelium parvum* are known to infect animals. However, the true host(s) of *Mycelium geminigerum* remains to be determined. In our case, these viruses might have infected the mongoose and the badger, or alternatively, might have arisen from fungi inhabiting their intestines, or they could also be derived from insects or plants as part of the diet of the badger and the mongoose. Therefore, since the true host cannot be determined yet, the nomenclature of these novel viruses should be addressed cautiously.
dependent RNA polymerase (RdRp) responsible for its RNA replication and RNA2 (1.2 kb) encodes for a capsid protein [31]. Nodaviruses are classified by the ICTV (International Committee on Taxonomy of Viruses) according to the genetic diversity of the RNA2 segment [34]. In the fecal material of the otter we identified a partial RNA1 of a novel putative Nodavirus (1.7 kb), which was most closely related to the recently discovered Mosinovirus, isolated from mosquitoes, sharing 43 % similarity on the amino-acid level (Fig. 2). Adopting the convention of naming based on Schuster and colleagues for Mosinovirus virus (mosquito nodavirus), we have tentatively named the virus Lunovirus (Lutra lutra nodavirus) [35]. As the RNA2 of Lunovirus was not found, most likely due to the fact that it is highly divergent and could not be detected by similarity searches in current viral databases, we should be reluctant to suggest a final classification. However, based on the large divergence of the Lunovirus RNA1 with the RNA1 of other nodaviruses, it seems likely that the Lunovirus is a novel member of the Nodaviridae family.

Conclusions

In the three healthy carnivores analyzed, viral sequences belonging to the Caudovirales order of bacteriophages were also detected, as previously reported [12]. Furthermore, our study showed that even healthy wildlife seems to harbor many divergent viral communities that deserve to be explored further to expand our current knowledge and databases. From the limited data available from the fecal virome studies from an otter (Lutra lutra) and badger (Meles meles) of Bodewes and van der Brand [12, 13], the virus families discovered in our study are...
completely different, possibly resulting from different diets, or indicating a large unexplored area of the ‘virome space’. Also the fact that the viruses discovered are highly different from viruses available in databases, might explain the difficulty in finding viruses by regular PCR screening, as previously reported [11]. These novel viruses reported in this study are likely derive from the diet, as the Nodavirus from the otter is likely to be from fish and the gemycircularviruses from insects, which are part of the animals diets. Viral discovery can be challenging because novel viruses, as seen in this study, can be quite divergent and their classification and true host

Table 1 Motifs of the Rep from the novel gemycircularviruses and reference gemycircularviruses

| Virus name                                      | Size (nt) | Nonanucleotide motif | Motif I | Motif II | GRS | Motif III | Walker-A | Walker-B |
|------------------------------------------------|----------|----------------------|---------|----------|-----|-----------|----------|----------|
| Mongoose feces-associated gemycircularvirus a  | 2089     | TATAAATAC            | LLTYA   | HLH5FID  | DFDVDGCHPNVSPTH | YACKD | GPSRMGKT | VFDDI    |
| Mongoose feces-associated gemycircularvirus b  | 2189     | TATAAATAC            | LFTYS   | HYHVFDV  | RKFDVDFHPNIVPSL | YATKD | GRSKGTGKT | VFDDI    |
| Mongoose feces-associated gemycircularvirus c  | 2124     | TAAATTAC             | LFTYS   | HLHAFVD  | RKFDVDFHPNITST  | YATKD | GPSRTGKT | VFDDI    |
| Mongoose feces-associated gemycircularvirus d  | 2251     | TAAATTAT             | LLTYA   | HLHCFVD  | RVFDVDFHNPISPR  | YAIKD | GRSTGKT  | VLDOD    |
| Badger feces-associated gemycircularvirus      | 2113     | TAAACTAT             | LLTYA   | HLHAIFVH | TVFDVAGHPNISPF  | YAIKD | GPSRVGKT | VFDDI    |
| MSSII2.225 virus                               | 2259     | TAAATTAT             | LLTYA   | HLHAFVD  | RADFDVDFHPNISPF | YAIKD | GKRSLSCS | IFDDF    |
| Fecal associated gemycircularvirus 1a          | 2197     | TAAATTAT             | LLTYA   | HLHAFVD  | DVFDFVGRHPNIVPSY | YAIKD | GDTRLGKT | VFDDM    |
| Faecal associated gemycircularvirus 4           | 2224     | TAAATTAT             | LLTYA   | HLHAFCD  | DVFDFVGRHPNIEAR | YAIKD | GDTRLGKT | VFDDM    |
| Fecal associated gemycircularvirus 5           | 2187     | TAAATTAT             | LVTYP   | HLHFCFD  | DVFDFVGRHPNISRF | YACKD | GDALTGKT | VDIID    |
| Cassava-associated circular DNA virus           | 2220     | TAAATTAT             | LLTYA   | HLHCFID  | DVFDFVGRHPNIPSW | YAIKD | GDSRGKT  | IFDDI    |
| Dragonfly-associated circular DNA virus-2       | 2236     | TAAATTAT             | LVTYP   | HLHCFAD  | DVFDFVGRHPNIPQST | YAIKD | GESENGT  | IFDDI    |
| Mosquito VEM virus SDBVL G                      | 2238     | TAAATTAT             | LLTYA   | HFAHLFD  | RFWDIAGRHPNIAVRG | YAIKD | GPSRGTGK | VFDDI    |

Fig. 2 Maximum-likelihood phylogenetic tree of the RNA1 of several Nodaviridae and Lunovirus. The tree represents viral members of the Nodaviridae and the partial RNA1 of the Lunovirus identified in the otter (Bootstrap values even or greater than 70 are shown). Viruses represented with an asterisk (*) have been currently recognized as Alphanodaviruses and viruses represented with a number sign (#) have been recognized as Betanodaviruses by the ICTV [34].

Golden pompano nervous necrosis virus
Tiger grouper Nervous Necrosis Virus
Sea bass Iberian betanodavirus
Epinephelus tauvina nervous necrosis virus
Redspotted grouper nervous necrosis virus
Senegalese sole Iberian betanodavirus
Tiger puffer nervous necrosis virus
Atlantic cod betanodavirus
Barfin flounder nervous necrosis virus
Dicentrarchus labrax betanodavirus
Striped Jack nervous necrosis virus

Alphanodavirus HB-2007/CHN
Lunovirus
Striped Jack nervous necrosis virus

Pariacoto virus*
Mosinovirus
Pieris rapae virus
Nodamura virus*
Convertnecrosis nodavirus
Boolarra virus*
Black beetle virus*
Flock house virus*
determination can be difficult. In this regard, replication associated proteins have shown to be conserved, and the best strategy to create alignments. It is very interesting to see the ubiquity of different circular virus species found nowadays due to the availability of next-generation sequencing. Screening of larger groups of animals and species will help to increase our knowledge of viruses circulating in wild animals.

**Methods**

**Sample collection**

Fecal samples were collected from a badger (*Meles meles*), a mongoose (*Herpestes ichneumon*) from a rescue center and an Eurasian otter (*Lutra lutra*) from a zoo upon their arrival in 2011 at the wildlife center “Parque Biológico da Serra da Lousã” in Coimbra district, Portugal. This collection was part of the quarantine assessment program applied to newly introduced animals in the center. Samples were kept at −80 °C until further processing.

**Sample preparation**

Ten percent fecal suspensions were homogenized for 1 min at 3000 rpm with a MINILYS homogenizer (Bertin Technologies) and filtered consecutively through 100 μm, 10 μm and 0.8 μm membrane filters (Millipore) for 30 s at 1250 g. The filtrate was then treated with a homemade buffer (1 M Tris, 100 mM CaCl₂ and 30 mM MgCl₂) and a cocktail of Benzonase (Novagen) and Micrococcal Nuclease (New England Biolabs) at 37 °C for 2 h to digest free-floating nucleic acids. RNA and DNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions but without addition of carrier RNA to the lysis buffer. First and second strand synthesis and random PCR amplification for 25 cycles were performed using a slightly modified Whole Transcriptome Amplification (WTA) Kit procedure (Sigma-Aldrich), with a denaturation temperature of 95 °C instead of 72 °C to allow for the denaturation of dsDNA and dsRNA. This modification leads to the amplification of both RNA and DNA. A size selection after library synthesis was performed using a 0.7 ratio of Agencourt AMPure XP beads (Beckman Coulter, Inc.). WTA products were purified with MSB Spin PCRapace spin columns (Stratagene) and were prepared for Illumina sequencing using the KAPA Library Preparation Kit (Kapa Biosystems). Fragments ranging from 350–600 bp were selected using the BluePippin (Sage Science) according to the manufacturer’s instructions. Libraries were quantified with the KAPA Library Quantification kit (Kapa Biosystems) and sequencing of the samples was performed on a MiSeq 2500 platform (Illumina) for 301 cycles (150 bp paired ends). Each sample was attributed a total of 2 million paired end reads. Mongoose feces associated gemycircularvirus a, b, c and d yielded 262, 295, 37,644, and 356 reads respectively. The Badger feces associated gemycircularvirus yielded 88 reads and the RNA2 of the Nodavirus 20 reads.

**Genomic and phylogenetic analysis**

Raw reads were trimmed for quality and adapters using Skewer [36] and were de novo assembled into contigs using SPAdes [37]. Scaffolds were classified using a tBLASTx search against all complete viral genomes in GenBank using an e-value cut-off of 10⁻⁴ [38]. Scaffolds with a significant tBLASTx hit were retained and used for a second tBlastx search against the GenBank nucleotide database using an e-value of 10⁻⁴ [38]. Open reading frames (ORF) were identified with ORF Finder analysis tools and the conserved motifs in the amino acid sequences were identified with HHMER [39]. Amino acid alignments of the viral sequences were performed with MAFFT version 7 [40] using the –auto option. Maximum likelihood phylogenetic trees were constructed in MEGA6.0 [41], using JCC (best substitution model) with 500 bootstrap replicates. Potential intron acceptor and donor sites from the novel gemycircularviruses were identified manually. Using the method above we were able to retrieve the five complete gemycircularvirus and partial sequence of the RNA2 of the Lunovirus. Presence of the discovered novel viruses was then confirmed by PCR and Sanger sequencing using the original extracts. Gemycircularviruses’ primers were designed in the cap-sid gene, covering the complete circular genome and the nodavirus primer pair targeted the RNA2 sequence found. All sequences from the novel viruses were submitted to GenBank [KP263543, KP263544, KP263543-KP263548, KP263546, KP263547, KP263548].

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

NCN conceived, designed and performed the experiments, analyzed the data, drafted the manuscript. MZ and EH performed data analysis and contributed to manuscript drafting. HL was involved in the experiment performance and data analysis. JRM collected the samples and drafted the manuscript. JM conceived and designed the study and contributed to data analysis and manuscript drafting. All authors read and approved the final manuscript.

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References

1. Morse SS, Mazet JA, Woolhouse M, Parrish CR, Carroll D, Karesh WB, et al. Prediction and prevention of the next pandemic zoonosis. Lancet. 2012;380(9836):1556–65. doi:10.1016/S0140-6736(12)61684-5.

2. Wang LF. Discovering novel zoonotic viruses. N S W Public Health Bull. 2011;22(5):6113–7. doi:10.1071/PH110078.

3. Temmann S, Davoust B, Berenger JM, Raoult D, Desnues C. Viral metagenomics on animals as a tool for the detection of zoonoses prior to human infection? Int J Mol Sci. 2014;15(6):10377–97. doi:10.3390/ijms150610377.

4. Cutler SJ, Fooks AR, van der Poel WH. Public health threat of new, reemerging, and neglected zoonoses in the industrialized world. Emerg Infect Dis. 2010;16(1):1–7. doi:10.3201/eid1601.081467.

5. Al-Tawfiq JA, Zumla A, Memish ZA. Travel implications of emerging infections. Curr Top Microbiol Immunol. 2007;315:325–44.

6. Yugo DM, Meng XJ. Hepatitis E virus: foodborne, waterborne and zoonotic transmission. Int J Environ Res Public Health. 2013;10(1):4503–37. doi:10.3390/ijerph100104507.

7. Wang LF, Eaton BT. Bats, civets and the emergence of SARS. Curr Top Microbiol Immunol. 2007;315:325–44.

8. Li W, Shi Y, Zu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2003;305(5438):676–9. doi:10.1126/science.1118391.

9. Middleton D. Hendra Virus. Vet Clin North Am Equine Pract. 2013;29(3):579–89. doi:10.1016/j.cvceq.2013.08.004.

10. Duarte MD, Henriques AM, Barros SC, Faguilha T, Mendonca P, Carvalho P, et al. Snapshot of viral infections in wild carnivores reveals ubiquity of parvovirus and susceptibility of Egyptian mongoose to feline panleukopenia virus. PLoS One. 2013;8(3):e59399. doi:10.1371/journal.pone.0059399.

11. Oliveira M, Sales-Luís T, Duarte A, Nunes SF, Carneiro C, Tenreiro T, et al. First assessment of microbial diversity in faecal microflora of Eurasian otter (Lutra lutra Linnaeus, 1758) in Portugal. Eur J Wildl Res. 2008;54:245–52. doi:10.1007/s10344-007-0137-8.

12. Bodewees R, Ruiz-Gonzalez A, Schapendonck CM, van den Brand JM, Osterhaus AD, Smits SJ. Viral metagenomic analysis of feces from small wild carnivores. Virology. 2014;419:1189. doi:10.1186/1742-420X-11-89.

13. van den Brand JM, van Leeuwen M, Schapendonck CM, Simon JH, Haagmans BL, Osterhaus AD, et al. Metagenomic analysis of the viral flora of pine marten and European badger feces. J Virol. 2012;86(4):2360–71. doi:10.1128/JVI.06419-11.

14. Ge X, Li J, Peng C, Wu L, Yang X, Wu Y, et al. Genetic diversity of novel myco-like DNA viruses discovered in the faecal matter of various animals. Virus Res. 2013;177(2):199–16. doi:10.1016/j.virusres.2013.08.008.

15. Kim HK, Park SJ, Nguyen VG, Song DS, Moon HJ, Kang BK, et al. Identification of a novel single-stranded, circular DNA virus from bovine stool. J Gen Virol. 2012;93(Pt 3):635–53. doi:10.1128/JVI.00788-08.

16. Reuter G, Bosse A, Delwart E, Pankovics P. Novel circular single-stranded DNA virus from turkey feces. Arch Virol. 2014;160(9):1405–23. doi:10.1007/s00705-014-2002-5.

17. Phan TG, Kapusinszky B, Wang C, Rose RK, Lipton HL, Delwart EL. The fecal viral flora of wild rodents. PLoS Pathog. 2011;7(9):e1002218. doi:10.1371/journal.ppat.1002218.

18. Nishiyama S, Dutia BM, Stewart JP, Meredith AL, Shaw DJ, Simmonds P, et al. Identification of novel anelloviruses with broad diversity in UK rodents. journal.ppat.1002218.

19. Ge X, Sherry DL, Wang L, Xing X, Wu Y, et al. Genetic diversity of novel circular ssDNA viruses in bats in China. J Gen Virol. 2011;92(Pt 11):2646–53. doi:10.1099/vir.0.037838-0.

20. Sikorski A, Dayaram A, Martins M, Ware J, Krabberger S, Stanton D, et al. Diverse circular ssDNA viruses discovered in droppings (Odobenus rosmarus). J Gen Virol. 2012;93(Pt 1):74–86. doi:10.1099/vir.0.037838-0.

21. Li W, Shi Y, Zu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2003;305(5438):676–9. doi:10.1126/science.1118391.

22. Pyka M, Fischer S, Rothmeier K, Vargova J, Dolezel J, Stiller M, et al. A novel parvovirus and susceptibility of Egyptian mongoose to feline panleukopenia virus. PLoS One. 2013;8(3):e59399. doi:10.1371/journal.pone.0059399.

23. Bodewees R, van der Geest J, Haagmans BL, Osterhaus AD, Smits SJ. Identification of multiple novel viruses, including a parvovirus and a hepevirus, in feces of red foxes. J Virol. 2013;87(13):7758–64. doi:10.1128/JVI.00568-13.

24. Ng TF, Chen LF, Zhou Y, Shapiro B, Stiller M, Heintzman PD et al. Preservation of viral genomes in 700-y-old caribou feces from a subarctic ice patch. Proc Natl Acad Sci U S A. 2014. doi:10.1073/pnas.140429911.

25. King AM, Adams MJ, Carstens EB, Lefkowitz EJ. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. London: Elsevier Academic Press; 2011. p. 1061–89.

26. Yu X, Li B, Fu Y, Jiang D, Ghabrial SA, Li G, et al. A geminivirus-related DNA virus with similarities to Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1. Arch Virol. 2014;159(6):1527–31. doi:10.1007/s00705-013-1890-5.

27. Shetty M, Maiti B, Shivakumar Santhosh K, Venugopal MN, Karunasagar I. Betanodavirus of marine and freshwater fish: distribution, genomic organization, diagnosis and control measures. Indian J Virol. 2012;23(2):114–23. doi:10.1007/s13337-012-0089-8.

28. Schuster S, Zirkel F, Kurb K, van Cleef KW, Drosten C, van Rij JP, et al. A Unique Nodavirus with Novel Features: Mosinovirus Expresses Two Subgenomic RNAs, a Capsid Gene of Unknown Origin, and a Suppressor of the Antiviral RNA Interference Pathway. J Virol. 2014;88(22):13477–54. doi:10.1128/JVI.02144-14.

29. Jiang H, Lei R, Ding SW, Zhu S, Skewer a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. BMC Bioinf. 2014;15:82. doi:10.1186/1471-2105-15-82.

30. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455–77. doi:10.1089/cmb.2012.0021.

31. Achtzuf SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403–10. doi:10.1016/0022-2836(90)90360-2.

32. Finn RD, Clements J, Eddy SR. HHMMER web server: interactive sequence similarity searching. Nucleic Acids Res. 2011;39(Web Server issue):W29–37. doi:10.1093/nar/gkr367.

33. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30(4):702–9. doi:10.1093/molbev/mst010.

34. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9. doi:10.1093/molbev/mst197.