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No Virological Evidence for an Influenza A - like Virus in European Bats

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Summary

New members of the influenza A virus genus have been detected recently in bats from South America. By molecular investigations, using a generic real-time RT-PCR (RT-qPCR) that detects all previously known influenza A virus subtypes (H1–H16) and a newly developed RT-qPCR specific for the South American bat influenza-like virus of subtype H17 a total of 1571 samples obtained from 1369 individual bats of 26 species from Central Europe were examined. No evidence for the occurrence of such influenza viruses was found. Further attempts towards a more comprehensive evaluation of the role of bats in the ecology and epidemiology of influenza viruses should be based on more intense monitoring efforts. However, given the protected status of bats, not only in Europe, such activities need to be embedded into existing pathogen-monitoring programs

Keywords

Influenza; infectious disease; surveillance; bats

From a virologist's view, bats seem to be a 'treasure trove' as they host a plethora of previously undescribed viruses some of which harbour considerable zoonotic disease

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Samples collection:
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Sample analyses:
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Primer design and positive control preparation: Centers for Disease Control and Prevention, Atlanta, USA

potential (Calisher et al., 2006). Bats (order *Chiroptera*) account for approximately one-fourth of all mammalian species, with more than 1150 bat species worldwide. Many chiropteran species show migratory behaviour, and some share habitats with humans or with livestock. Special characteristics such as long lifespan (up to 35 years), high population density (up to several million individuals in one colony), multiple-species communities, clustered roosting behaviour, autumn swarming, propensity to develop persistent infection with certain viruses and high spatial mobility may contribute to their capacity to perpetuate various virus species and occasionally transmit them to other vertebrates (Calisher et al., 2006). Currently, metapopulations of aquatic wild birds represent the known primary natural reservoirs of influenza A viruses (IAV, Olsen et al., 2006). The recent discovery of the genome of a new influenza A-like H17 virus (IAV-like) in three little yellow-shouldered bats (*Sturnia lilium; Phyllostomidae* family) in Guatemala drew attention to bats as putative host species or even reservoirs of unknown influenza viruses (Tong et al., 2012). However, only very limited data on influenza virus surveillance in old world bats are available (Muhldorfer et al., 2011). This prompted us to analyze a collection of samples obtained from a diversity of European bat species. The samples had mainly been collected in the framework of surveillance efforts for selected viruses, for example lyssa- and paramyxoviruses, in Germany, Romania and the Czech Republic between 2002 and 2013 (mainly 2010–2012).

To this end, 1571 samples (1069 oropharyngeal swabs, 284 faecal samples or rectal swabs and 218 urine samples) from 1369 individual bats from 31 different sampling locations in Central Europe have been examined using a novel real-time reverse transcription polymerase chain reactions (RT-qPCR). Samples originated from 26 different bat species of the *Verpertilionidae* and *Rhinolophidae* families comprising *Barbastella barbastellus* (35), *Eptesicus nilsonii* (14), *Eptesicus serotinus* (64), *Hypsugo savii* (2), *Miniopterus schreibersii* (2), *Myotis alcathoe* (22), *Myotis bechsteinii* (121), *Myotis brandtii* (132), *Myotis capaccinii* (13), *Myotis daubentonii* (214), *Myotis emarginatus* (3), *Myotis myotis* (60), *Myotis mystacinus* (44), *Myotis nattereri* (76), *Myotis oxygnathus* (1), *Nyctalus leisleri* (64), *Nyctalus noctula* (129), *Pipistrellus nathusii* (105), *Pipistrellus pipistrellus* (44), *Pipistrellus pygmaeus* (132), *Plecotus auritus* (77), *Plecotus austriacus* (3), *Rhinolophus euryale* (2), *Rhinolophus ferrumequinum* (2), *Rhinolophus hipposideros* (6) and *Vespertilio murinus* (5). Conventional cotton swabs or rayon swabs were used for sample collection. A cold chain was maintained until arrival at the laboratory thereafter samples were stored at −80°C or directly subjected to manual (QiAmp viral RNA kit, Qiagen, Hilden, Germany; TriFast, Peqlab, Erlangen, Germany) or automated RNA extraction (Freedom EVO 3000, Tecan, Crailsheim, Germany). RNA was then examined by two different RT-qPCRs using the Superscript III One-Step RT-PCR kit (Invitrogen, Karlsruhe, Germany) or the AgPath One-step RT-PCR kit (Ambion). Based on the published M gene sequence of the Guatemalan bat influenza-like virus, a new and specific RT-qPCR was developed in our laboratory and validated based on IAV-like M gene plasmid-derived DNA (Table 1). This RT-qPCR detected with high sensitivity solely the Guatemalan bat influenza-like virus but no further influenza A virus subtypes. In addition, a generic IAV M gene RT-qPCR was employed (Fereidouni et al., 2012), which included an external inhibition control. The latter RT-qPCR has been validated to detect a very broad range of influenza A viruses of subtypes H1–H16 (Fereidouni et al., 2012) but did not react with the Guatemalan bat influenza-like virus (Tong

et al., 2012). Thus, use of the two RT-qPCRs in parallel ensured that presence in bat samples of known influenza A virus-like M gene sequences is detected and differentiated.

Despite analysing a large number of samples originating from different European bat species collected from a broad geographical range, no positive sample was found in either of the RT-qPCRs, covering all 16 IAV subtypes and the IAV-like H17 one. Hence, circulation in European bat species of orthomyxoviruses detectable by the two RT-qPCRs appears to be uncommon. This is supported by previous investigations on dead-found bats in Europe (Muhldorfer et al., 2011). Although it cannot be fully excluded that geographical sampling locations, season and tissue targets may have been suboptimal and infectious phases could have been missed, it should be noted that the Guatemalan bat influenza-like virus was detected in a sample size comparable to or even lower than that of many of our study species.

Evidently, the IAV-like virus was isolated from a member of the bat family *Phyllostomidae,* and it is possible that this particular virus is restricted to this New World bat family or even to this species. Due to phylogenetic differences of host and/or pathogen, this particular virus might not occur in European bats and, hence, was not detected. European bat species of the *Verspertilionidae* and *Rhinolophidae* tested in our study may nevertheless harbour yet unknown IAV-like viruses that may go undetected with the assays applied. Orthomyxoviruses are well known for their low level of sequence conservation; they also have a history of host switching, and therefore, their phylogeny is not linked to the evolution of their hosts.

Generally, a comprehensive assessment of a putative role of bats in the ecology and epidemiology of influenza viruses would require a more intense monitoring of larger sample sizes, accompanying serological investigations and virus isolation attempts. Given the protected status of bats in Europe and beyond, such efforts should be linked to already established pathogen-monitoring schemes and can only work in close cooperation with bat biologists. In this line, reporting the lack of detection of influenza A viruses of subtypes H1–H16 and of the Guatemalan bat influenza-like virus is deemed an important contribution.

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Impacts

- Bats have been identified a ‘treasure trove’ of new mammalian virus species.
- New influenza A virus subtypes have been detected in bats from South America.
- No evidence, by molecular investigations, for the occurrence of such influenza viruses was found in bats from Central Europe.

Table 1

RT-qPCR primers used in this study specific for bat influenza-like virus M gene.

RT-qPCR	Primer designation	Primer sequence (5′→3′)
BatIV-M1	M_bat_fw	TGA GCA TCT TAA CAG AGG TTG A
	M-bat_rv	CAA TGG GGA CAG TAT GGG TCT
	M_bat_FAM	FAM- CCA TCA GGG CCT CTA AAA GCT GAC A -BHQ1

The thermal cycler parameters for the RT-qPCR assay were 50° for 30 min, 94° for 2 min, followed by 42 cycles (94° for 20 s, 51° for 20 s and 68° for 20 s) using the Superscript III One-Step RT-PCR kit (Invitrogen) or 45° for 10 min, 95° for 10 min and 42 cycles (95° for 15 s, 55° for 20 s and 72° for 30 s) using the AgPath One-step RT-PCR kit (Ambion).