Molecular Methods for Studying Bat-Associated Pathogens

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bats PCR viruses diseases

1. Introduction

Bats (order Chiroptera) are a group of mammals with unique anatomy and physiology, which predispose them to fulfill a variety of ecological functions [1]. Known as the only mammals capable of sustained flight [1], bats occur ubiquitously except in Antarctica [2].

The impact of bats as reservoirs of zoonotic viruses has been defined in numerous studies [3][4][5][6]. Infectious pathogens, especially those that infect more than one species, are a complex subject that has recently captured the attention of many researchers. This impact is underlined by the fact that about 75% of zoonoses are spread to humans from wildlife [7][8][9][10][11][12][13]. One of the most important bat-borne zoonoses is bat rabies. Human–bat interactions are now the source of the majority of locally acquired human lyssavirus infections in many high-income countries without hematophagous or ‘vampire’ bat species [14]. On the other hand, experimental European bat lyssavirus (EBLV) infections in other mammals have revealed only low susceptibility of foxes by the intramuscular route [15]; an experimental EBLV infection of sheep resulted only in a peripheral abortive infection without neurological signs [16]. As for known or supposed natural infections, evidence suggests that Hendra virus and Nipah virus may have been transmitted from bats to other mammals beside humans [17][18][19]. Hendra virus was not highly contagious in experimental conditions [20].

Besides their role in emerging infectious diseases (EID), bats also fulfil indispensable roles in the functioning of the ecosystem [1][21]. While nectarivorous species are important pollinators of fruit-bearing trees and frugivorous bats play a role in the process of reforestation [22][23][24], insectivorous species are significant predators of insects [1][21][25]. Whenever bat populations experience a higher mortality rate, the decline in numbers may affect the whole ecosystem, even economics [26]. The guano of bats is rich in nitrogen and is used as fertilizer [27]. In several countries, bats are listed as species protected by local laws. Therefore, research of bat-related EID is ruled by

ethical committees; specific bat biological sample availability is limited in some cases, as non-invasive sampling methods must often be preferred in these species [28].

2. Molecular Methods in Bat EID Research

Molecular methods, sequencing and bioinformatics have recently become irreplaceable tools in EID research and even outbreak prediction. As the number of available studies implies, bats seem to be linked more to research concerning RNA viruses than DNA viruses [29][30]. Although several DNA viruses were detected in bats [12][31][32], these do not seem to have a significant impact on bat populations; neither do these viruses seem to be the etiological agent of a zoonotic disease outbreak.

RNA isolation, transcription and a variation of PCR [33] are therefore used quite often as first-choice methods in virus detection in bats, and sequences of the most common viruses are available in databases like GenBank.

2.1. PCR Leading to Discoveries

A standard PCR protocol followed by sequencing of purified amplicon is a versatile way of both identifying well-known viruses and finding new viruses. Examples of the relevance of this approach are studies similar to Straková et al. [34], who identified a novel hantavirus in bat liver tissue, performing a total RNA extraction (QIAamp viral RNA Mini Kit or Qiazol/triazol method) from internal organ tissues collected from dead individuals and proceeding to screen tissue samples for hantavirus RNA presence and sequencing of PCR amplicons (from a broad-spectrum RT-PCR). To determine the entire genomic sequence of this novel virus, IonTorrent HTS analysis was performed.

Cadar et al. [35] identified Usutu virus RNA in bat brain tissue by performing both total DNA and RNA extraction from tissues, then subjecting extracted nucleic acids to RT-PCR. Amplicons were sequenced directly and these sequences were used for phylogenetic analysis. Similar to Straková et al. [34], a complete genome sequence of the bat Usutu virus was then obtained from bat brain tissue using a set of primers designed from multiple comparisons of Usutu virus genomes available in databases [35].

A novel bat-borne hantavirus was detected in samples of bat lung tissue by Arai et al. [36], using a heminested L-segment primer set and a nested S-segment primer set. Their results might be indicative of a host-switching event [36], which might help understand the ecology of these viruses.

Luo et al. [37] collected 1044 bat brains and 3532 saliva swab samples to search for molecular evidence of rhabdoviruses. For initial screening, they used a previously described combined real-time reverse transcription PCR (RT-qPCR) that includes a probe-based RT-qPCR for pan-rabies virus detection and another pan-lyssavirus RT-qPCR [37]. Their effort led to the discovery of six new rhabdoviruses, the sequences of which were determined by next-generation sequencing and confirmed by Sanger sequencing. One of the tentative rhabdovirus species identified in this research clustered with two insect-related viruses; the researchers did not exclude a possible role for arthropods in the life cycle of the identified bat viruses [37]. Although these rhabdoviruses were considered

unlikely to present a high risk of spillover events, further information about transmission and shedding of these viruses in bats is needed to determine their zoonotic potential [37].

A wide range of rhabdoviruses was discovered in bats and bat parasites by Aznar-Lopez et al. [38], who performed a nested RT-PCR on 1488 oropharyngeal swabs from bats.

2.2. Coronaviruses Are Found Abundantly Using Non-Invasive Methods

Bat guano was successfully used as a sample for coronavirus detection in a long-term study performed by Lo et al. [39]. The researchers obtained 512 fecal samples over the course of 4 years. RNA was isolated from these samples and carried out a nested PCR for coronavirus detection. Analysis of the sequences obtained in their study revealed that the detected coronaviruses belonged to the genera *Alphacoronavirus* and *Betacoronavirus*, some of which were grouped with the SARS-like coronavirus clade [39]. Using non-invasive sampling methods, such as guano collection, can lead to significant discoveries in bat-borne virus research; identification of non-invasive or less invasive samples such as saliva, urine or feces is also a key element in terms of bat conservation [37].

2.3. Modern Sequencing Methods and Viral Diversity

Sanger's sequencing method [40] and its more modern modifications belong to the first generation of sequencing methods. This method has been reported to still be the most accurate. It is widely accepted that NGS variants need to be validated with the gold standard Sanger sequencing technique prior to reporting, even though both the costs and turnaround time of this approach are considerable [41]. Another question needing an answer is how much can be concluded about the reservoir status of the bat even after successfully confirming the presence of a pathogen nucleic acid fragment in the samples. To fully confirm the reservoir status of a species, much more data is required; however, depending on the location of the detected pathogen fragment in the body of the host, the direction of further research into the virus–host relationship can be determined.

It was pointed out that traditional Sanger sequencing can only be applied to individual samples (or a low number thereof), which makes the method too painstaking for processing complex samples, especially for large-scale studies [42]].

In the literature, the term next-generation sequencing (NGS) is often used to describe sequencing platforms other than those based on the Sanger method (pyrosequencing, sequencing by synthesis, ligation and two-base coding) [43]. NGS, also known as massively parallel or deep sequencing, is characterized by the ability to sequence millions of short DNA fragments in parallel [44]. NGS has proved to be a very efficient method to determine the virome of mammals, including bats, such as the extensive and highly efficient study performed by Wu et al. [12] in which a broad range of viruses, most of them novel, were identified in swab samples from 4440 bats. Metagenomics, defined as the direct genetic analysis of genomes contained with an environmental sample [45], has led to important findings, some of which encompass novel and/or potentially zoonotic viruses in bats [46][47][48][49][50]. Library preparation, being an important part of metagenomics, has also been pivotal in the research of full-genome sequencing of novel bat-associated viruses [51][52]. Wu et al. [12] used a series of sequence-independent RT-PCR,

sequence-based PCR and specific nested PCR amplification methods, along with viral library construction and NGS, to analyze the viral community in the sampled bat species. Wu et al. [12] stated that the purpose of their study was to survey the ecological and biological diversities of viruses residing in these bat species, to investigate the presence of potential bat-borne zoonotic viruses and to evaluate the impacts of these viruses on public health. Recently, metagenomics has found a potential use in diagnostics [53] and surveillance [54].

2.4. In Silico Analyses May Reveal Bases for Further Research

While it has long been known that the eukaryotic genome contains endogenous retroviruses, it was surprising to discover that sequences of RNA viruses that do not make a DNA intermediate and do not usually enter the nucleus are also present in eukaryotic genomes [55][56]. A useful collective term reflecting their fragmentary nature, EVE (endogenous viral elements), has been coined by Katzourakis and Gifford [57]; Holmes [56] uses this term to refer to all endogenous viruses regardless of taxonomy.

Using an initial PCR screening and phylogenetic analyses, Horie et al. [58] demonstrated that bats of the genus *Eptesicus* carry an inheritable endogenous bornavirus-like L (EBLL) element in their genome. Representatives of the genus *Eptesicus* occur in a wide geographical area including the northern hemisphere within Europe, Asia and the Americas [59]. These findings provide novel insights into the co-evolution of RNA viruses and mammalian species [58].

Taylor et al. [60] performed first an in silico screening of NIRV (non-retroviral integrated RNA viruses) in bat sequences; they further tested the presence of integrated copies of DNA-based filovirus sequences in the two species with the highest copy number, the wallaby species *Macropus eugenii* and the bat species *Myotis lucifugus*. They designed PCR primers from the mammalian genomic sequence belonging to the longer identical sequences and performed amplification of these segments in individuals of these species other than those used in existing genomic projects. The sequence from *Macropus eugenii* had only one mutation compared to the sequence from the mentioned genome project [60]. Taylor et al. [60] subsequently tested samples of bats of *Myotis lucifugus* and *Eptesicus fuscus* for the presence of these sequences. In all cases, the similarity of the new sequences was consistent with the hypothesis of integration of a filovirus-like copy of DNA into mammalian genomes. This laboratory finding supported their hypothesis expressed after an in silico examination of the genomic database; the phylogenetic analysis and sequencing performed in this work is consistent with the hypothesis of integration of filoviral elements into mammalian genomes [60]. Phylogenetic evidence suggests that the direction of transfer was from viral to mammalian genome [60].

Integrated viral elements have been found not only in bats but also in the genome of arthropod vectors [57][60].

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