UNIVERSITY OF PÉCS

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Bat related viral zoonoses in Algeria

Ph.D. Thesis

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1. Introduction

The chiropteran order is the most diverse and largest order of mammals with roughly 1423 recognized species and exceptional powered flight and echolocation ability (Han et al., 2015). They have a broad, geographical distribution range, apart from the polar regions, extreme desert climates, and a few oceanic islands (Irving et al., 2021). Based on phylogenetic analysis, bats can be traced back to 52.5 million years ago. This allowed their co-evolution with several viruses, therefore making them natural reservoir hosts (Wynne & Wang, 2013). Furthermore, to date, all performed studies on bats' immunity, metabolism, and mitochondrial dynamics point out their adaptation to flight's demands and consequences. In addition to their altered innate immune response which balances immune tolerance mechanisms and host defense strategies, hypothetically contributing to their lengthy lifespans and diminished incidence rates of cancer, in addition to their exceptional status as an asymptomatic viral reservoir (Banerjee et al., 2020; Gorbunova et al., 2020; Irving et al., 2021).

Zoonoses are defined as diseases naturally transmitted from vertebrate animals to humans. Approximately, 70% of the emerging zoonotic disease events originated from wild animals mainly those with a taxonomically diverse range of species such as bats (Hassell et al., 2017; Jones et al., 2008). Moreover, encroachment into natural animal habitats due to the global expansion of the human population gave rise to an increased contact rate among animals and humans (Hassell et al., 2017). While globalization, travel, and trading ease the spread and ensure hard to control outbreaks occurrence despite the implementation of well-established mitigation measures (Sabin et al., 2020). Unsurprisingly, in the last decade, several serious emerging viral diseases were associated with bats. For instance, the severe acute respiratory syndrome (SARS) outbreak declared in China in 2002 was linked to the Chinese horseshoe bats (reservoir) following many investigations (Shi & Hu, 2008). Similarly, the subsequent coronavirus outbreak: the Middle East Respiratory Syndrome (MERS) first reported in 2012 and allegedly originated among bats (Han et al., 2016). Lastly, in 2019 the new emergent SARS-CoV-2 caused a pneumonia outbreak in Wuhan China, and thereafter a worldwide pandemic. Regardless of the blurred route by which SARS-CoV-2 spilled over to humans, most hypotheses sustain bats as natural reservoirs (Prince et al., 2021; Temmam et al., 2021). In parallel, based on intensive molecular analyses regarding viruses and their chiropteran hosts, numerous viral families were reported from bats, this include but not limited to *Lyssaviridae, Astroviridae, Caliciviridae, Coronaviridae, Flaviviridae, Hepeviridae, Picornaviridae, Arenaviridae, Filoviridae, Hantaviridae, Nairoviridae, Orthomyxoviridae, Paramyxovirdae, Parvoviridae, Adenoviridae, Herpesviridae and Hepadnaviridae* (Chen et al., 2020; Letko et al., 2020). Nevertheless, many aspects such as zoonotic potential. spillovers events, evolution, and much more all remain poorly understood. This inquires more research to fill the knowledge gap necessary to aid in the outbreak and pandemic preparedness efforts (Aarestrup et al., 2021; Simpson et al., 2020).

In Algeria, located in North Africa with an area of 2.4 million km^2 and 45 million inhabitants, there is a vast bat diversity arising from the various biotopes (Ahmim, 2018). despite their broad distribution and presence in urban areas near humans, bats are still poorly studied, particularly as viral reservoirs. Remarkably, no other

references are available about bat-related viral zoonoses except our studies regarding picornaviruses (Zeghbib et al., 2019) and coronaviruses(Zeghbib et al., 2021).

2. Aims of the study

- Bat sampling from caves located in urban areas near humans.
- Large-scale virus discovery among the collected samples and accurate host identification using applied genomics.
- Evolutionary and comparative analyses of the characterized viruses.
- Investigation of the novel SARS-CoV-2 pandemic in Algeria via in silico analyses.
- Characterization of multiple disease introductions and the transmission patterns related to the Algerian outbreak.
- Description of Algerian nucleotide and amino acid mutations with the prediction of their effect on the corresponding protein.
- Perform molecular tracing and point out the missing unsampled data.
- Assessment of the implemented mitigation measures through statistical analysis

3. Materials and methods

Sample collection

Between 2016 and 2018, guano samples were collected from Aoukas and Melbou caves in Bejaia city located on the Algerian coast. Moreover, in 2018, additional sampling was performed in the Ibn-Ziad cave situated in the city of Constantine. A total of 97 samples were harvested and transported in a dry shipper for further analysis.

Bat guano samples preparation and nucleic acid extraction

Samples were homogenized using 500 µl of 1X PBS and two pieces of 2.0-2.5 mm diameter glass beads (Kisker Biotech GmbH & CO., Germany) for 60 sec at maximum speed by means of a Minilys® personal homogenizer (Bertin Corp., USA). The Nucleic acid extraction was conducted with strict adherence to the manufacturer's recommendations from 200 µl of supernatant using the GeneJET Viral DNA/RNA Purification Kit (Thermo Fisher Scientific., USA) or Genaid viral nucleic extraction kit II (Geneaid Biotech Ltd., Taiwan) dependent upon availability. The extracted nucleic acid was eluted in 50 µl of nuclease-free water, then stored at -80° C.

Polymerase Chain Reaction (PCR) methods

We used several oligonucleotides and PCR types for viral-specific nucleic acid detection, molecular identification (barcoding), or full genome recovery. For fragments up to 800 bp, Reverse transcription-polymerase chain reactions (RT-PCR) were achieved using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany), whereas longer fragments (>1000 bp) were obtained with the SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Thermo Fisher Scientific, USA). Similarly, Conventional PCRs were performed using

the GoTaq® G2 Flexi DNA Polymerase Kit (Promega, USA) (fragment ≤ 800 bp). While the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) was utilized for long amplicons (>800 bp).

Cloning and Sanger Sequencing

Regarding bat coronaviruses, the resultant PCR amplicons were ligated into pGEM-T Easy vector (Promega, USA) and the recombinant plasmids were used for Escherichia coli JM109–competent cells (Promega, USA) transformation in full adherence to the manufacturer's protocols. For blue/white selection of the recombinant colonies, the transformed cells were plated on Luria-Bertani medium (LB; Sigma Ltd.) dishes containing 100μg/ml ampicillin, 0.5mM IPTG, and 40μg/ml X-Gal and incubated overnight at 37° C. The positive clones undergo a colony PCR using pGEM-T Easy Vector-specific primers. Subsequently, a purification step was conducted with the Geneaid Gel/PCR DNA fragment kit (Geneaid Biotech Ltd, Taiwan). The BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) was employed for sequencing PCR. Samples were processed on an ABI Prism 310 DNA Sequencer (Applied Biosystems, USA).

Next-Generation sequencing with Ion Torrent PGM and Illumina

Prior to library preparation, we combined low-speed centrifugation (12,000 g/10 min), filtration using Sartorius filter tubes (Sartorius, Germany), and enzymatical digestion [Micrococcal nuclease (Thermo Fisher Scientific, USA) and Benzonase (Sigma-Aldrich., USA)] to increase viral reads when performing metagenomics. Additionally, to target RNA viruses, we performed a Sequence-Independent, Single-Primer-Amplification (SISPA) protocol. For the IonTorrent platform, libraries were constructed with the use of NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit (New England Biolabs, USA) and the IonTorrent Xpress barcode adapters. Subsequently, a clonal amplification was performed using the Ion PGM Template kit on a OneTouch v2 instrument (Life Technologies, USA) and positive beads were enriched via an Ion OneTouch™ ES pipetting robot (Life Technologies, USA). The sequencing was achieved on a 316 chip (Life Technologies, USA). In parallel and in support of Illumina, sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs). Library preparation was followed by sequencing on the Illumina NextSeq with 2 × 150 bp read length (Illumina). For data analysis, CLC Genomics Workbench version 9.0 (http://www.clcbio.com) was employed for raw sequence readings, trimming, and quality control. While Taxonomic binning was achieved with Diamond v0.8.3.

Rapid Amplification of cDNA-Ends by Polymerase Chain Reaction (3' RACE-PCR)

This was performed concerning the bat picornavirus genome. Using SuperScript III Reverse Transcriptase kit (Invitrogen, USA) and 2nd Generation RACE kit (Roche, Switzerland), first-strand cDNA was obtained in combination with Oligo dT-Anchor Primer a designed sequence-specific primer (1/3′ race). Thereafter the cDNA amplification was pursued with PCR Anchor Primer (Roche, Switzerland) and a second sequence-specific primer (2/3′ race). The resultant amplicons were first visualized on 1.2% agarose gel, then sliced and cleaned using the Geneaid Gel PCR DNA fragment extraction kit (Geneaid, Taiwan). Sequencing was achieved with Ion Torrent

(Life Technologies, USA. Finally, the CLC Genomics Workbench (http://www.clcbio.com/) was used for de novo sequence assembly and reference mapping.

Sequence editing, Temporal signal assessment, phylogenetic and phylogeographic analysis

In reference to the bat picornavirus, BtPVs analysis the dataset comprised 70 RNA dependent RNA polymerase (RdRp) sequences and 29 bat-related cytochrome B sequences. Sequences were aligned in MAFFT web server (Katoh et al., 2002). Then, non-molecular clock Bayesian phylogenetic trees were constructed using MrBayes v3.2.4 software (Ronquist et al., 2012) for both BtPVs and their hosts. Each analysis operated for 10 million generations (25% were discarded as burn-in) and sampled every 1,000 generations. the resultant trees were then visually edited using iTOL (Letunic & Bork, 2016).

Similarly, the bat coronavirus dataset encompassed 24 partial helicase genes and 806 partial RdRp sequences. First, sequences were aligned in the MAFFT web server using the default parameters (Katoh et al., 2002). Thereafter, in the IQTREE webserver, both substitution model selections and maximum likelihood phylogenetic trees with ultrafast bootstrapping were implemented.

Lastly, the SARS-CoV-2 analysis comprised two datasets; a global one contained 95 genomes (29 Algeria, 66 worldwide) and a local dataset constituted only of the Algerian sequences. Prior to time-calibrated phylogenetic trees inference, the temporal signal within both datasets was assessed using TempEst as formerly discussed (Rambaut et al., 2016). Thereafter, BEAST package 1.10.4 was utilized for molecular clock phylogenetic tree reconstruction regarding the global pandemic under the GTR+I and a lognormal uncorrelated relaxed clock. With consideration to population growth, an exponential growth coalescent model was chosen (Drummond et al., 2006; Suchard et al., 2018). In parallel, for the Algerian pandemic, we performed phylogenetic and both discrete and continuous phylogeographic analyses under the following parameters: the time-calibrated phylogenetic tree was inferred under the GTR+I substitution model with a lognormal uncorrelated relaxed clock and a skyline plot coalescent model to draw the population dynamic. For the discrete phylogeography, we combined a Bayesian stochastic search variable selection (BSSVS) and a standard symmetric substitution model regarding the discrete diffusion process to infer significant transitions between Algerian cities based on Bayes factor generation (BF). Whereas, the continuous diffusion model based on the Brownian diffusion assuming a homogeneous dispersal rate over the phylogeny, which can infer ancestral states based on coordinates (latitude and longitude) was applied for the continuous phylogeographic diffusion analyses (Lemey et al., 2010). Thereafter, SpreaD3 v0.9.7 software was used to visualize the transmission routes and calculate the Bayes Factor (BF) (Bielejec et al., 2011).

Pairwise genetic distance

Using the MegAlign Pro program (DNASTAR v15.2.0) or MEGA v6 (Tamura et al., 2013), the number of nucleotide or amino acid changes was calculated among pairs of sequences.

Phylogeny-trait association analysis

For BtPiV, the degree of association between the phylogeny and sampling location, host genus, or host species was estimated using BaTS package (Parker et al., 2008).

Virus-host co-evolution analysis

The reconciliation program JANE v 4.0 (Conow et al., 2010) which reduces the frequency of different evolutionary events (co-speciation, duplication, host switch, loss, and failure to diverge) was used to evaluate the co-evolution between Picornaviruses and their corresponding chiropteran hosts.

Selection pressure and mutation analyses

This was performed with regard to the Algerian sequences when compared to the Chinese reference sequence. The selection pressure based on the ω ratio representing the rate of the non-synonymous mutation (Ka/dn) to the synonymous mutations (Ks/ds), according to Nei and Gojobori, was calculated for the following genes: *ORF1a, ORF1b, S, E, M, N, ORF3a and ORF8* using SNAP v2.1.1 (Bromberg & Rost, 2007; Masatoshi Nei & Takashi Gojobori, 1986; Yang & Bielawski, 2000). In parallel, the CoVsurver mutations App implemented in the GISAID database was employed to highlight both amino-acid and nucleotide mutations among the Algerian sequences (Elbe & Buckland-Merrett, 2017). Thereafter, the effect of amino acid changes on their corresponding proteins was predicted with the PredictSNP web server (https://loschmidt.chemi.muni.cz/predictsnp/, accessed on 1 August 2021).

Recombination analysis

Recombination events were detected either using the RDP4 software (Martin et al., 2015) (SARS-CoV-2) or based on incongruencies observed in phylogenies constructed from different genes (P1, P2, P3 of BtPiV).

Statistical analysis

Spearman coefficient was used among the *Picornaviridae* family to estimate the strength of the correlation among picornavirus diversity represented by the number of picornaviruses detected clusters and the number of *Picornavirus* species four each host genus. On the other hand, to assess the mitigation measures implemented regarding the SARS-CoV-2 pandemic containment in Algerian, the linear, exponential, and logarithmic trend lines were compared after the collection of the infected, recovered, and death cumulative cases. Thereafter, the best model was chosen based on the R^2 values. Subsequently, the correlation coefficient was calculated between the population density (calculated for each Algerian city) and the number of confirmed cases (within each city).

Haplotype network analysis

After discarding the partial Algerian SARS-CoV-2 genomes, a dataset comprising 84 sequences was subjected to the DnaSP v6.12.03 package for haplotype file generation (Rozas et al., 2017) and thereafter to POPART

software for Haplotype network analysis based on the country of origin via the median-joining network method with default setting (epsilon=0) (Bandelt et al., 1999; Leigh & Bryant, 2015).

4. Sum-up of the main results and their significance

With regard to the *Picoronaviridae* family:

- We were able to describe the first picornavirus genome from an Algerian *Miniopterus schreibersii* bat. The novel virus clustered within the *Mischivirus* genus with the Hungarian Mischivirus B sequences.
- We highlighted the major role of host-jumping events in the evolutionary history of BtPVs within their chiropteran hosts and the putative function of sympatry in increasing host-jumping events.
- Through the Phylogenetic-trait association analyses, we emphasized the partial association between both host genus and host species indicating that picornaviruses are passed between bats from different genera and species. In parallel, the partial association of the phylogeny with the large-scale sampling locations (continents) pointed out the virus dispersal among bat populations located in geographically distant areas probably eased by the bat's migratory ability.
- Based on the pairwise genetic distances, we could notice that BtPVs from the same host genus were very distinct and not closely related indicating multiple virus introductions to bats hypothetically due to co-roosting.
- We demonstrated both potential and significant recombination events among BtPVs hence indicating its potential role in both the diversity and the evolution of BtPVs.
- However, we encountered some drawbacks such as the unbalanced dataset (some genera and locations were oversampled, while others were undersampled), the length, and the number of BtPVs (short sequences and a small dataset) were major limitations in our investigation.

For Bat related *Coronaviridae*:

- We described four bat-related alpha coronaviruses and two beta coronaviruses from different bat species identified with DNA barcoding located in urban areas close to humans.
- Our beta coronaviruses clustered with a sequence that was 100% identical to the RATG13, the closest relative to *SARS-CoV-2*, which may indicate the relatedness of our sequences to the novel emerging coronavirus.
- We reported for the first time an alpha coronavirus from a Plecotus gaisleri bat
- We highlighted the cocirculation of an alpha coronavirus and a beta coronavirus within a *Rhinolophus ferrumequinum* bat which may trigger potential recombination events and a subsequent new variant emergence.
- Nevertheless, the short length of the sequences and the absence of literature regarding Coronaviruses in *Plecotus gaisleri* hampered both the accurate analyses and reliable conclusions.

In support of the SARS-CoV-2 pandemic:

- We could estimate the starting date of the Algerian pandemic based on the time-calibrated phylogenetic analysis.
- Based on the phylogenetic and the haplotype network analyses we could emphasize both multiple disease introductions and missing unsampled data.
- We could sketch an overview about the disease spread in Algeria referring to both discrete (migration between cities) and continuous (diffusion in the whole country with the inference of ancestral locations based on GPS coordinates) phylogeographic analysis.
- We estimated the evolution of different coding genes under a negative selection which is not surprising for such types of genes to keep their function. However, the dataset comprised only 29 Algerian sequences (plus the reference sequence) sampled at the beginning of the pandemic, hence with more data the results will be different.
- We characterized the mutation pattern on both nucleotide and amino acid levels. Based on this we could perform a molecular tracing and identify both imported and exported mutations. Additionally, we could predict deleterious amino acid replacements (change the protein function) and neutral replacements as well.
- We assessed the effectiveness of the mitigation measures implemented for disease containment (overall good measures) with reference to the linear growth of the confirmed, recovery and death cases. Moreover, we identified some cities with high population density reporting low confirmed cases and vice versa despite the unsurprising positive correlation between the city's density and the number of cases. This emphasizes the role of the population awareness in disease transmission.
- As previously reported, the number and the length of sequences were the main inconveniences for the performed analyses.

5. References

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6. Publications

Articles related to the thesis

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Zeghbib S. Genetic and evolutionary characterization of a novel picornavirus in Algerian *Miniopterus schreibersii* bats *5th International Congress on Infectious Diseases* Berlin, Germany, March 01-02, 2018.

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