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RESEARCH LETTER

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Detection of a *Merbecovirus* with potential ACE2 usage in France

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ABSTRACT

A novel *Merbecovirus*, designated Cam-2023, has been identified in *Pipistrellus pipistrellus* in France through non-invasive surveillance. Phylogenetic analysis demonstrates that Cam-2023 belongs to a *Merbecovirus* clade previously associated with ACE2 usage in closely related viruses detected in the Netherlands and Russia. While the receptor usage of Cam-2023 remains to be functionally validated, sequence similarities within the Spike protein, particularly the receptor-binding domain, suggest a putative association with a *Merbecovirus* clade previously associated with ACE2 usage. This discovery broadens the known host diversity of this lineage and extends its geographical range to Western Europe. Our findings highlight the importance of continuous surveillance in European bat populations to better characterize the distribution and zoonotic potential of such high-risk coronaviruses.

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KEYWORDS Coronavirus; detection; genome; bats; merbecovirus

Introduction

Betacoronaviruses can cause serious diseases in humans, such as *severe acute respiratory syndrome* (SARS-CoV and SARS-CoV-2), as well as *Middle East respiratory syndrome* (MERS-CoV). Although most MERS-CoV use the cell receptor DPP4, other *Merbecovirus* such as NeoCoV (KC869678), PDF-2180 (NC034440), MOW15-22 (ON325306) and PnNL2018 (OQ405399) use the ACE2 (Angiotensin-Converting Enzyme 2) receptor [1,2]. ACE2 is a key coronavirus entry receptor that has been implicated in the emergence of several zoonotic coronaviruses. Although none of the ACE2-associated *Merbecoviruses* have been detected in humans to date, their molecular characteristics suggest a potential to cross species barriers, highlighting the importance of continued surveillance. Our study reports the identification of a new *Merbecovirus* in *Pipistrellus pipistrellus* in Camargue (southern France) (Figure 1).

Materials and methods

This virus was identified during non-invasive surveillance conducted in the Camargue delta during 2023 [4]. Sampling was non-invasive and conducted under the supervision and authorization of the Tour du Valat Research Institute; no formal animal ethics approval was required as no animals were handled. During these campaigns, samples of fecal droppings from live bats nesting in the immediate vicinity of human dwellings (behind shutters, under roofs) were collected. These were stored in RNAlater immediately after collection, and nucleic acids (RNA) were extracted in the laboratory. The presence of CoVs was investigated using a semi-nested PCR (Polymerase Chain Reaction) protocol targeting the *RdRp* (RNA-dependent RNA polymerase) gene, following the exact parameters described [5]. Specifically, the first round utilized the Pan-CoV R1 and F1 primers (40 cycles, Ta = 50°C), and the second round used Pan-CoV R2 and F2 (40 cycles, Ta = 52°C). The specificity of the reaction was systematically validated using positive and negative controls, and all resulting amplicons were confirmed by sequencing. It revealed the co-circulation of several coronavirus lineages, mainly *Alphacoronavirus*, among bats in the region [4]. While our year-long surveillance revealed the co-circulation of several coronavirus lineages,

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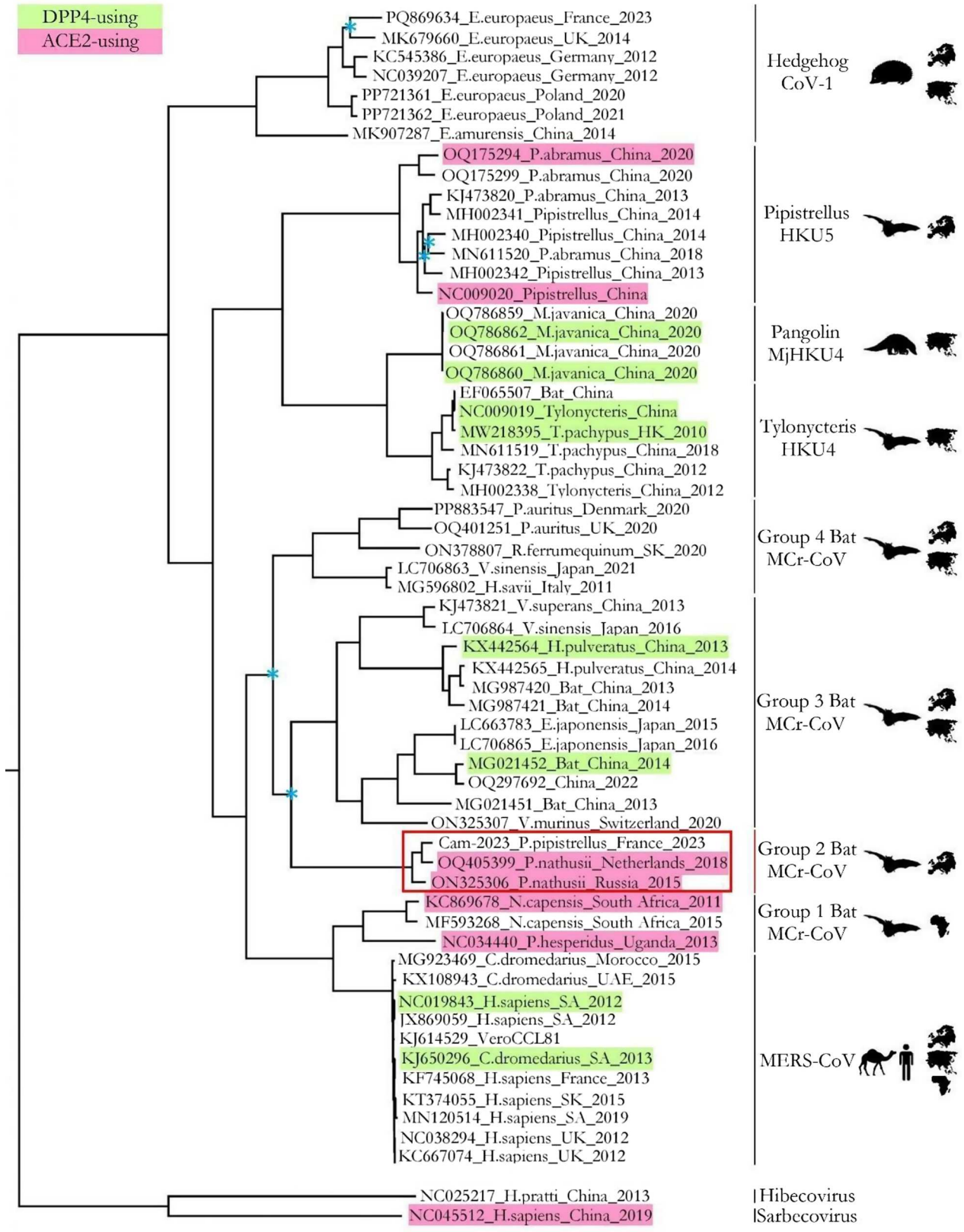


Figure 1. Maximum likelihood phylogenetic tree based on the complete genome of Merbecoviruses. Green background indicates sequences using DPP4 as receptors, pink background indicates clades previously associated with ACE2 usage; information from previous studies (1–3]. Host species and continents of occurrence are represented using pictograms for each group. The tree is rooted using one Hibecovirus and one Sarbecovirus sequence. All bootstraps are greater than 80, except those marked with a blue asterisk (*).

primarily *Alphacoronavirus*, *Merbecovirus* was exclusively identified in three guano samples collected from the same site in Camargue on May 11, 2023; however, the samples were collected from three spatially distinct locations on the aluminium foil, suggesting that they could originate from three different bats. These three samples were the only *Merbecovirus*-positive detections throughout the entire 2023

surveillance campaign. After deep sequencing, analyses enabled three genomes to be assembled, though only one was complete due to the inherent challenges of working with environmental samples. Although two sequences are partial (for one sequence we obtained 45% of the genome, less than 2% for the other), a global alignment revealed the presence of two single nucleotide polymorphisms (SNPs), indicating some viral diversity within the site. These partial sequences were used strictly for confirmatory purposes and diversity assessment; they were excluded from the primary phylogenetic reconstruction and comparative genomic analyses to ensure the highest level of accuracy. Its sequence has been deposited in Genbank (accession number = PX975258). The identification of bat species was assessed using genotyping based of a 400 bp fragment of the mitochondrial 12S rRNA gene [5].

Sequences were aligned using MAFFT v7.526 in reference-guided mode (– add) with the following options: – keeplength – reorder – anysymbol – nomemsave – adjustdirection. The reference sequence used for alignment was KC667074.1 Merbecovirus MERS2c Homo sapiens 2012 England/Qatar. The resulting alignment, comprising 61 taxa, was inspected for quality and ambiguous regions were trimmed as necessary. The two partial genomes were processed using the same bioinformatics pipeline but were only used for site-specific SNP identification. Due to their insufficient coverage, they were not integrated into the final alignment used for phylogenetic inference. Only the complete Cam-2023 genome was utilized for the calculation of pairwise similarities and the construction of the maximum likelihood tree. Phylogenetic relationships among the sequences were inferred using IQ-TREE v2.3.6. The nucleotide substitution model GTR was specified, and branch support was assessed with 1,000 ultrafast bootstrap replicates (-bb 1000). The resulting maximum likelihood tree was visualized and annotated using FigTree v1.4.4. A full list of the genomes used, along with their GenBank accession numbers, is provided (Supplementary – Table 1).

Pairwise sequence similarities were assessed using the *distmat* program from the EMBOSS suite (v6.6.0), applying the formula $S = \frac{m}{npos + gaps \times gap_penalty}$, where m is the number of identical positions, *npos* is the number of aligned positions excluding gaps, and *gaps* is the number of positions with a gap weighted by the associated penalty. The complete genomes of three sequences (Cam-23, OQ405399 and ON325306) were compared with the MERS-CoV (KC667074) sequence as an alignment reference. The highest similarity was recorded between Cam-23 and OQ405399 (Netherlands sequence) (97.32%, corresponding to a distance of 0.0268), followed by Cam-23 and ON325306 (Russian sequence) (96.04%, distance 0.0396) and finally OQ405399 and ON325306 (95.98%, distance 0.0402). These values indicate that Cam-23 and OQ405399 are more closely related to each other than to ON325306, which is consistent with the topology inscribed in the phylogenetic tree (Figure 1). These three sequences obtained similarity scores between 75.61% and 75.68% with the MERS-CoV sequence. Using the same method, the spike sequences of these three viruses were compared to the MERS-CoV Spike sequence. The highest similarity was obtained between the spike sequences from Russia and the Netherlands (89.07%, corresponding to a distance of 0.1093, followed by the French sequence and the Dutch sequence (81.47%, corresponding to a distance of 0.1853), and finally, the French and Russian sequences (80.98%, corresponding to a distance of 0.1902). We also compared the RBD (Receptor Binding Domain) portions of these 3 sequences, it appears that the French sequence shares 97.63% similarity with the Dutch sequence, and 90.24% with the Russian sequence, while the two sequences between them have a similarity of 89.67% (Supplementary – Figure 1).

A maximum likelihood phylogenetic tree based on the complete genome sequences of 58 *Merbecovirus* shows that the Cam-2023 virus clusters with PnNL-2018 and MOW15-22 with a bootstrap support value of 100 (Figure 1). These three viruses form a distinct clade within the *Merbecovirus* subgroup, separate from human MERS-CoV strains and other African *Merbecovirus* using the ACE2 receptor (PDF-2180 and NeoCoV). Indeed, our Cam-2023 sequence is closely related to European viruses found in *Pipistrellus nathusii* in Russia [6] and the Netherlands [7], which is quite distant from the one formed by human MERS-CoV on the one hand, and African strains found in Uganda in *Pipistrellus hesperidus* [8], and in South Africa in *Neoromica capensis* [9] on the other hand. This tree topology suggests both a spread of this specific *Merbecovirus* lineage among European pipistrelles, widely distributed between Russia and Western Europe, and at least one interspecies transmission event between *Pipistrellus nathusii* and *Pipistrellus pipistrellus*. The close genetic proximity between these three genomes, as well as their circulation among pipistrelle bats, reinforce the hypothesis of trans-European circulation.

Results and discussion

These results highlight the expansion of the host range of this *Merbecovirus* clade to this bat species, *Pipistrellus pipistrellus*. It is one of the most common and widespread bat species in Europe, living from the Iberian Peninsula to western Russia and North Africa [10]. This small insectivorous bat frequently roosts in buildings, including houses and other anthropogenic structures [11], and may undertake seasonal movements or migrations of several hundred kilometers across

Europe [12]. In addition, *P. pipistrellus* can form mixed colonies or shares roosts with other bat species, which may facilitate interspecific viral transmission. This species is also known to harbor *alpha* – and *beta-coronaviruses*, although no *Merbecovirus* has ever been detected in Europe [4,13–14].

While most studies on *Merbecovirus* are conducted in Asia or Africa, the detection of Cam-2023 represents the first identification in France of a *Merbecovirus* belonging to a clade previously associated with ACE2 usage. Although no affinity testing has been performed to date, based on the Spike sequence, and in particular the RBD region of Cam-2023, the proximity of its sequence to that of PnNL-2018 and MOW15-22 suggests a putative association with a clade previously associated with ACE2 usage, which warrants further functional validation. Although none of the Merbecoviruses belonging to this clade previously associated with ACE2 usage have been detected in humans to date, this receptor has been implicated in the emergence of several zoonotic coronaviruses, supporting the relevance of continued surveillance. Although this work does not demonstrate an imminent risk of emergence of this virus in the human population, continued long-term surveillance will allow us to monitor its evolution and spread in this bat species or in other European bat species. The detection of Cam-2023 at a single time point, despite a continuous year-long monitoring effort that successfully identified other coronaviruses, underscores the potentially sporadic or seasonal nature of *Merbecovirus* circulation in these populations. However, the screening of Cam-2023 using high sensitive Real-Time PCR assay and specific probe and primers in all samples will be required to evaluate more accurately the level of Cam-2023 circulation. Furthermore, the limited recovery of full genomic sequences is a direct consequence of the non-invasive nature of guano sampling, where RNA degradation is frequent. Consequently, while our study provides a valuable "snapshot" of viral presence in France, further long-term studies and using more specific and sensitive methods are required to define its precise prevalence and ecological dynamics. Since May 2023, regular biannual monitoring in the Camargue has been implemented to track the virus's evolution and potential spatial dissemination, and the analyses are currently ongoing.

Conclusion

In conclusion, the presence of *Merbecovirus* in southeastern France suggests that its circulation could be much more widespread than previously estimated and probably underdetected on the continent, demonstrating the importance of including European bats in the surveillance of coronavirus diversity and evolution. While our genomic data strongly support the classification of Cam-2023 within a *Merbecovirus* lineage phylogenetically associated with ACE2 usage in related viruses, further experimental studies using pseudotyped viruses will be required to definitively confirm its receptor tropism.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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