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RESEARCH

Bartonella Infection in Fruit Bats and Bat Flies, Bangladesh

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Abstract

Bats harbor diverse intracellular *Bartonella* bacteria, but there is limited understanding of the factors that influence transmission over time. Investigation of *Bartonella* dynamics in bats could reveal general factors that control transmission of multiple bat-borne pathogens, including viruses. We used molecular methods to detect *Bartonella* DNA in paired bat (*Pteropus medius*) blood and bat flies in the family Nycteribiidae collected from a roost in Faridpur, Bangladesh between September 2020 and January 2021. We detected high prevalence of *Bartonella* DNA in bat blood (35/55, 64%) and bat flies (59/60, 98%), with sequences grouping into three phylogenetic clades. Prevalence in bat blood increased over the study period (33% to 90%), reflecting an influx of juvenile bats in the population and an increase in the prevalence of bat flies. Discordance between infection status and the clade/genotype of detected *Bartonella* was also observed in pairs of bats and their flies, providing evidence that bat flies take blood meals from multiple bat hosts. This evidence of bat fly transfer between hosts and the changes in *Bartonella* prevalence during a period of increasing nycteribiid density support the role of bat flies as vectors of bartonellae. The study provides novel information on comparative prevalence and genetic diversity of *Bartonella* in pteropodid bats and their ectoparasites, as well as demographic factors that affect *Bartonella* transmission and potentially other bat-borne pathogens.

Keywords Hippoboscoidea · Nycteribiidae · Pteropodidae · Ectoparasites · Vector-borne pathogen

Introduction

Bats (Mammalia: Chiroptera) represent the second-most diverse order of mammals (after Rodentia) and inhabit various ecological niches on every continent except Antarctica [1]. Bats have been of increasing interest to pathogen research owing to their role as hosts of medically important viruses (e.g., lyssaviruses, coronaviruses, henipaviruses, filoviruses) [2]. However, the prevalence and dynamics of bacterial pathogens in bats are less studied despite reports of genera with zoonotic potential (e.g., *Rickettsia*, *Bartonella*, *Borrelia*, *Coxiella*) in bats and their ectoparasites [3, 4]. Moreover, bacterial infections in bats may provide insights into the general health of bat populations and perhaps even the drivers of viral spillover from bats to other species [5–9].

Bacteria in the genus *Bartonella* have been reported from numerous wildlife species, and are agents of Carrion's disease, trench fever, cat scratch disease, neuroretinitis, and endocarditis in humans [10]. *Bartonella* spp. have been detected in multiple families of bats, as well as the hematophagous arthropods that parasitize them, including bat flies (Diptera: Nycteribiidae, Streblidae), ticks (Ixodida: Ixodidae, Argasidae), and mites (Mesostigmata: Spinturnicidae, Macronyssidae) [11–14]. Little is known about the temporal dynamics of *Bartonella* infection in bats and their ectoparasites. *Bartonella* infections can be seasonal in rodent populations due to the timing of reproduction and the intensity of ectoparasite infestation [15], but these factors have not been investigated in longitudinal surveys of bats. Beyond

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questions about *Bartonella* dynamics in bats, *Bartonella* diversity is poorly characterized in some bat families and regions, so there is limited understanding of how *Bartonella* species are shared across related bat hosts or populations living in sympatry [16]. Despite the family Pteropodidae being one of the most species-rich bat families, reports characterizing prevalence of *Bartonella* in these bats and their bat flies exist from only a few locations [14, 17–21]. Additional data on *Bartonella* diversity across bat families would help to understand the host specificity and geographic range of *Bartonella* species, and the frequency of interspecies contact and transmission of bat-borne infections.

The Indian flying fox (*Pteropus medius*) is a large pteropodid species native to the Indian subcontinent. Previous studies have examined the role of *P. medius* as a reservoir of Nipah virus (*Paramyxoviridae: Henipavirus*) in this region, contributing to knowledge of the species' ecology and behavior over time [22]. However, rare detections of Nipah virus in these bats has constrained our ability to identify demographic and ecological factors that influence transmission patterns in bats. Relatively little is known about the hematophagous arthropods that parasitize these bats and their role in the transmission of *Bartonella* and other pathogens, but the generally high prevalence of *Bartonella* in bats may provide sufficient data to uncover factors that affect pathogen transmission generally. The aim of this pilot study was to investigate the prevalence and genetic diversity of *Bartonella* bacteria in *P. medius* and ectoparasitic bat flies in a longitudinally sampled population in Bangladesh, describing temporal changes in prevalence in relation to host demography and ectoparasite infestation, and confirm historical records of bat-bat fly species associations in *P. medius*.

Methods

Field Site

This work is part of a longitudinal study on viral transmission dynamics in *P. medius* bats across multiple sites in Bangladesh. This pilot study focuses on a subset of bats from one population in Faridpur District. The bat roost site was situated in a partially wooded, peri-urban area ~ 1.75 km west of the Faridpur city center in central Bangladesh (Fig. 1A). The bats were spread among 18–20 roost trees, including bamboo (species not identified), mahogany (*Swietenia mahagoni*), tamarind (*Tamarindus indica*), and wood apple (*Limonia acidissima*) within a village area near a road and several residential buildings. The roost had been occupied for at least 15 years prior to its destruction in September 2021, with an estimated population size varying from 963 to 3147 bats based on flyout counts (Fig. 1B). Bangladesh has four seasons with a pronounced period of heavy rainfall: winter (December–February), spring (March–May), monsoon (June–September), and post-monsoon (October–November).

Animal Capture

Pteropus medius individuals roost in dense aggregations (Fig. 2) at the tops of trees and are most easily captured in flight while leaving or returning to the roost. Custom-made mist nets of 10 m × 15 m were suspended between bamboo poles mounted at the top of trees near the bat roost. Nets were operated over several nights each month between 11 pm and 5 am as bats returned to the roost following foraging until the desired number of bats were captured (n = 20–30). To minimize stress on bats, nets were continuously monitored, and bats were immediately retrieved from the net after capture. All members of the field team wore personal protective equipment during capture and specimen sampling, including coveralls, respirators, safety glasses, nitrile gloves, disposable aprons, and leather welding gloves when restraining bats. Following capture, bats were held in cotton pillowcases for no more than 6 h before being released at the site of capture. Bat specimens were collected at a dedicated field laboratory near the capture site. Bats were anesthetized with isoflurane gas prior to collection of blood and ectoparasite specimens. In addition, the age class, weight, sex, body condition (poor, fair, or good based on visual inspection), reproductive status, and morphometrics from each bat were recorded. Age classes included pups (less than 4–6 months old), juveniles (approximately 4–6 months to 2 years old), or adults based on body size and the presence of secondary sexual characteristics [22]. A small microchip was inserted under the skin on the animal's back prior to release to facilitate identification of recaptured bats.

Sample Collection, Storage, and Shipment

Blood samples (< 125 µL) were collected from the brachial vein of bats onto Whatman FTA cards (Sigma-Aldrich, Munich, Germany) and stored at ambient temperature. If bat flies were visible on bat pelage, one or more individuals were collected and stored in a sterile cryovial with 1 mL of 70% ethanol. No attempt was made to census all ectoparasites on each bat, and there may have been mites, larval ticks, or fleas that were missed. Ectoparasite loads were not quantified on all bats, only the presence/absence of nycteribiids. Blood spots were collected from 144 bats (out of 154 captured), and 119 bat flies were collected from 111 bats in Faridpur between March 2020 and January 2021 (Table 1). The timing of this study coincided with COVID-19 lockdowns in Bangladesh, so no sampling was performed in April–August 2020. Ethanol was removed from sample tubes then shipped from icddr,b to Colorado State University

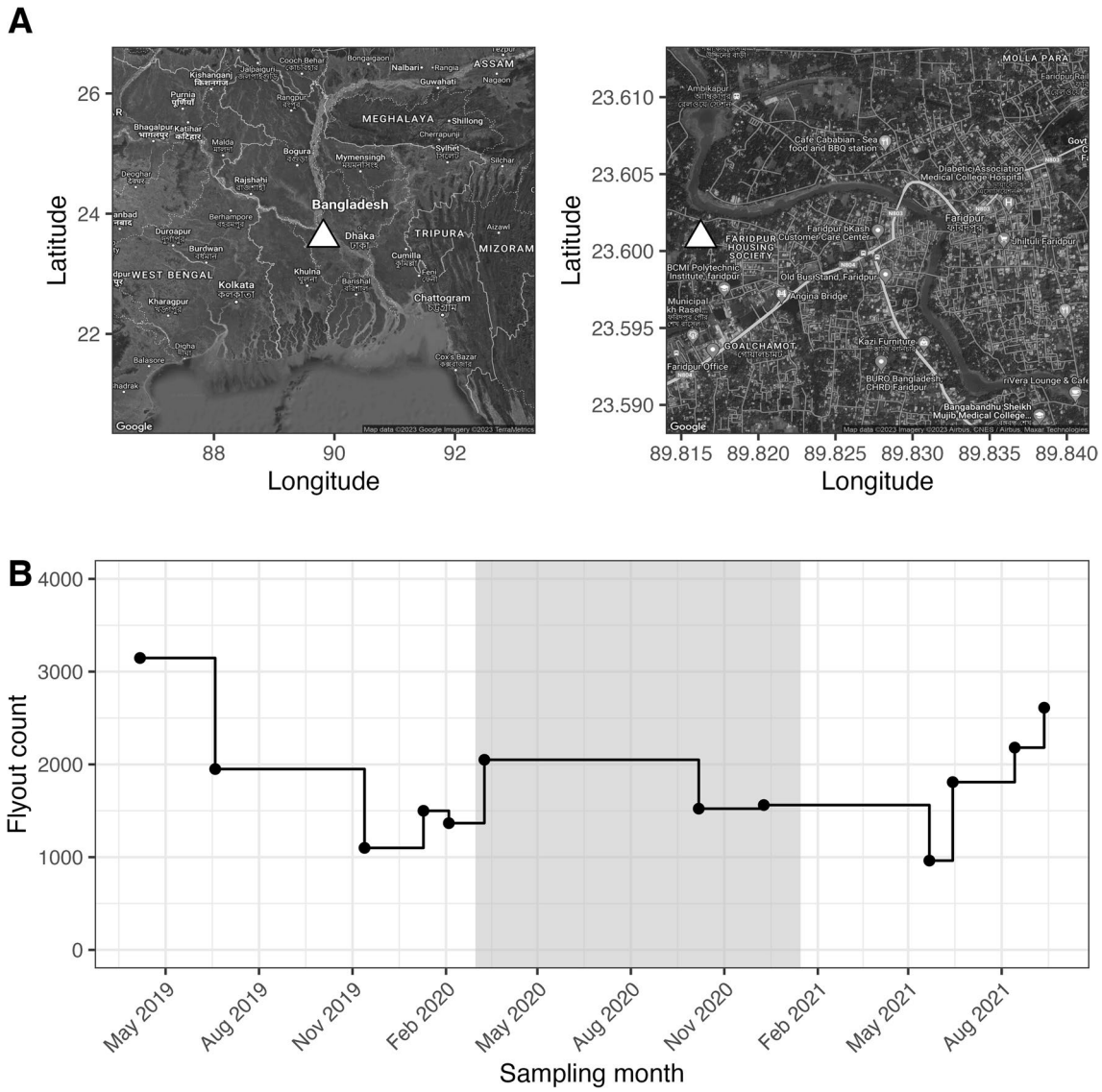


Fig. 1 Location of the *P. medius* bat roost (white triangle) in Faridpur, Bangladesh (**A**) and the flyout counts of bats residing in the roost (**B**). The sampling period discussed in this study, March 2020 to January 2021, is highlighted with a gray box in **B**

at ambient temperature and stored at −20 °C until processing. From the list of bats with paired blood spots and nycteribiids ($n = 124$), we used simple random sampling to select 10 bats from each sampling month between March 2020 and January 2021, yielding 60 pairs of blood and nycteribiid samples for analysis.

Identification of Bat Flies

Flies were placed in 99% acetone for 15 min and then air dried. Specimens were point mounted and morphologically identified under a microscope using available keys and illustrations [23]. Two voucher specimens were deposited in the C.P. Gillette Museum of Arthropod Diversity, Fort Collins, CO, USA, which does not use accession numbers. Additional specimens were photographed using a stereo microscope (Leica EZ4 W, Leica Microsystems, Milton Keynes, UK) (Fig. 3, inset).

DNA Extraction and PCR

One nycteribiid was selected from each selected bat sample and manually macerated prior to incubation in 1X DNA/RNA Shield (Zymo Research, Irvine, CA, USA) with 2% proteinase K and two silica beads for 12–18 h at 56 °C. Total nucleic acid was extracted using the Zymo *Quick*-DNA/RNA Viral Kit. Amplification of invertebrate 16S ribosomal RNA (rRNA) was used as a proxy for DNA integrity (forward

Fig. 2 *Pteropus medius* bats roosting in a tree, Bangladesh (photo credit: Ausraful Islam)

Table 1 Blood sample collection, and prevalence of nycteribiids on *P. medius* in Faridpur, Bangladesh, March 2020 to January 2021. Prevalence of nycteribiids was calculated as the proportion of bat individuals with ≥ 1 bat fly

Month	Season	Bats captured	Blood samples	Nycteribiids collected	Nycteribiid prevalence
March 2020	Spring	25	15	23	60% (15/25)
September 2020	Monsoon	27	27	16	60% (16/27)
October 2020	Post-monsoon	26	26	24	92% (24/26)
November 2020	Post-monsoon	23	23	19	83% (19/23)
December 2020	Winter	23	23	18	78% (18/23)
January 2021	Winter	30	30	19	63% (19/30)

primer (LR-J-13007): 5′-TACGCTGTTATCCCTAA-3′ and reverse primer (LR-N-13398): 5′-CGCCTGTTTATCAAA AACAT-3′) using existing protocols [24]. If nycteribiid DNA did not amplify, extraction was repeated a second time, and 16S rRNA amplification was confirmed prior to pathogen screening.

For DNA extraction from dried blood spots, five punches of equal dimension were removed from each blood spot using a 2-mm biopsy punch. If a blood spot was not large enough in diameter to collect five 2-mm punches, the maximum number of punches was taken (minimum 2 punches per bat). DNA was extracted from punches using the QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany). Punches were incubated in 280 μL Buffer ATL and 20 μL proteinase K for 60 min, vortexing every 10 min. The rest of extraction was completed following the manufacturer's protocol and eluted in 60 μL Buffer ATE. Following extraction, DNA integrity was assessed by amplifying mitochondrial cytochrome oxidase I (*COI*) using previously described methods [25].

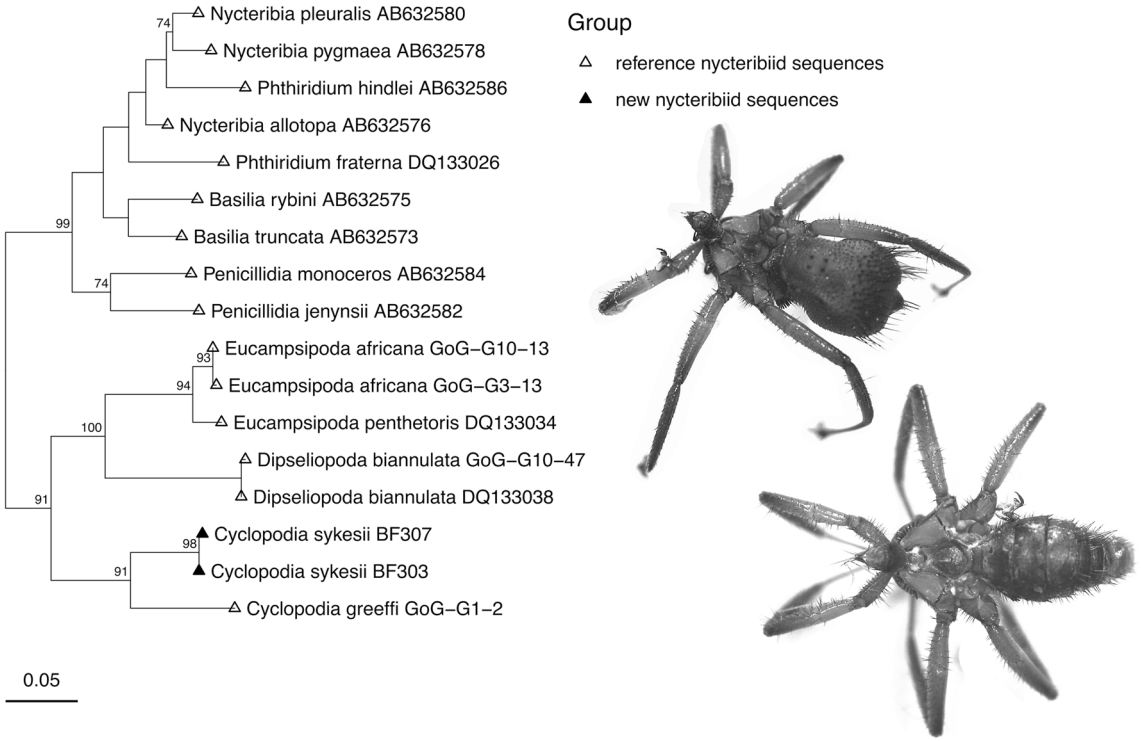


Fig. 3 Phylogenetic relationships between nycteribiids according to 16S rRNA sequences. Separate groups, including new sequences from Bangladesh, are indicated by distinct symbols. The maximum likelihood tree was inferred using a TIM + F + G4 model in IQ-TREE from a 424 bp alignment (new sequences from this study were 422 bp). Numbers next to nodes indicate the percent bootstrap support after 1000 replicates. Branch lengths are in units of substitutions per site. Inset: representative microscope photos of *Cyclopodia sykesii* (top, female; bottom, male; photo credit: Anna Fagre)

Samples with successfully amplified host DNA (16S rRNA for arthropod samples and *COI* for dried blood spots) were subjected to *Bartonella* genus-level consensus nested PCR targeting the citrate synthase gene (*gltA*) using modified methods [26]. The first round of PCR results in amplification of a 767 bp product (forward primer CS443f [27]: 5′-GCTATGTCTGCATTCTATCA-3′ and reverse primer CS1210r [28]: 5′GATCYTCAATCATTTCTTTCCA-3′), and the second round of PCR results in a 356 bp product (forward primer BhCS781.p [29]: 5′-GGGGACCAGCTC ATGGTGG-3′ and reverse primer BhCS1137.n [27]: 5′-AAT GCAAAAAGAACAGTAAACA-3′).

Sequencing and Phylogenetic Analysis

Amplicons were visualized using gel electrophoresis and cleaned using the QIAGEN QIAquick PCR Purification Kit prior to being submitted for Sanger sequencing at Azenta Life Sciences (formerly GENEWIZ, Chelmsford, MA, USA). Forward and reverse reads were visualized using SnapGene Viewer (GSL Biotech LLC, San Diego, CA, USA) and assembled using the *sangeranalyseR* package in R v4.2.2 [30, 31] with additional manual editing and trimming using GBlocks v0.91b [32]. A few *Bartonella* sequences with multiple peaks visible in the electropherogram were separated into major and minor sequences using the Mixed Sequences Reader tool [33]. Assembled sequences were inspected with the NCBI Basic Local Alignment Search Tool (BLAST) to confirm that *Bartonella* was amplified and to find closely matching sequences in the NCBI GenBank database. We created a database of *gltA* sequences from named *Bartonella* species, 16S rRNA sequences from nycteribiids, previous studies of bats and bat flies, and additional close matches from BLAST (Supplementary File, Tables S1 and S4). Amplified sequences and references were aligned with MAFFT v7 using the local, iterative method (L-INS-i) with default parameters [34]. Phylogenetic model selection and maximum likelihood tree estimation for each gene were performed with IQ-TREE v2 [35]. Trees were visualized using the *GGTREE* R package [36].

Statistical Analysis

Confidence intervals for nycteribiid and *Bartonella* prevalence were estimated using the Pearson exact method. Differences in *Bartonella* prevalence across demographic groups were tested using chi-square tests of proportions by sex, age class, and body condition, and a generalized linear model for

month with prevalence as binomial variable. All statistical tests used $\alpha = 0.05$ for determining significance.

Results

Identification of Bat Flies

Nycteribiids (Fig. 3, inset) were identified as *Cyclopodia sykesii* Westwood (1834) based on female morphology [23]. Males of *C. sykesii* and *Cyclopodia horsfieldi* are morphologically indistinguishable [23]. Previous records of *C. sykesii* list *P. medius* (formerly *P. giganteus*) as the primary host in India, Sri Lanka, and the Maldives [37]. Many of these records are over 60 years old and thus lack molecular data. We submitted mitochondrial 16S rRNA sequences from two *C. sykesii* to GenBank with accession numbers OQ401037 and OQ401038.

Detection Prevalence

The overall prevalence of nycteribiids on bats in the colony (i.e., the proportion of bat individuals with ≥ 1 bat fly) during the March 2020 to January 2021 sampling period was 72% (111/154). From the 60 paired blood/ectoparasite samples that were selected for analysis, amplification of invertebrate DNA targeting the 16S rRNA gene was successful in 100% of the nycteribiid samples ($n = 60$), and amplification of vertebrate DNA targeting the COI gene was successful in 91% ($n = 55$) of the blood spots. Nested PCR targeting the *gltA* gene of *Bartonella* resulted in a detection prevalence of 98% (59/60) in DNA from nycteribiids and 64% (35/55) in bat blood (Table 2). Infection status was congruent between some paired bats and bat flies: 35 pairs were both *Bartonella*-positive, 24 flies were positive with no detection in paired blood spots, and one pair was completely negative. Since there was little variation in *Bartonella* prevalence in bat flies across bats, we only examined differences in *Bartonella* prevalence in blood spots. There was no significant difference in prevalence between males (69%) and females (58%) ($\chi^2 = 0.34$, p value = 0.56). There was no significant difference in prevalence by body condition: good (70%) vs. fair (57%; $\chi^2 = -0.42$, p value = 0.46). The higher prevalence in adults (70%) vs. juveniles (36%) was borderline significant ($\chi^2 = -0.71$, p value = 0.08). Between March 2020 and January 2021, prevalence in bats monotonically increased from 33% to 90% ($z = 3.2$, p value = 0.001). This increase in prevalence (Fig. 4A) was concurrent with a small decrease in the number of bats in the roost (Fig. 1) and a shift in the age distribution of the roost (Fig. 4B), with a higher proportion of juvenile bats captured than adults in September–November 2020. This period also showed an increase in the prevalence of nycteribiids on captured bats (Fig. 4C), especially in juveniles.

Table 2 Prevalence of *Bartonella* in bat blood spots across demographic variables. The total denominator ($n = 55$) is based on the samples that were successfully extracted and were positive for mitochondrial COI

Sample group	Bat blood spots
Sex	
Female	58% (15/26)
Male	69% (20/29)
Age group	
Juvenile	36% (4/11)
Adult	70% (31/44)
Body condition	
Fair	57% (16/28)
Good	70% (19/27)
Month	
March 2020	33% (2/6)
September 2020	33% (3/9)
October 2020	50% (5/10)
November 2020	70% (7/10)
December 2020	90% (9/10)
January 2021	90% (9/10)

Phylogenetic Analysis

The phylogeny of nycteribiid 16S rRNA (Fig. 3) clustered the sequences from *C. sykesii* closest to *C. greeffi*, a congener that parasitizes straw-colored fruit bats (*Eidolon helvum*) in Africa [23]. A selection of sequences from other nycteribiid species supports the placement of *Cyclopodia* sequences within its nominal subfamily Cyclopodiinae along with the genera *Dipseliopoda*, *Eucampsipoda*, and *Leptocyclopodia*.

A total of 32 distinct *Bartonella gltA* genotypes were amplified from bats and bat flies and the genotypes grouped into three clades (Fig. 5) with high bootstrap support (≥ 99%). Ten genotypes in Clade A shared 94.55–99.72% identity among each other and were most closely related (95.08–99.24% identity) to *Bartonella* sequences previously detected in *Pteropus hypomelanus* and associated *C. horsfieldi* in Malaysia (GenBank accessions KY677753, KY677752, and JX416257). Clade A is also nested in a larger clade containing sequences from other bat families in Africa and Asia, with many from bats in the suborder Yinpterochiroptera (Pteropodidae, Hipposideridae, Rhinolophidae, and Rhinonycteridae) but a few others from Emballonuridae. Clade B consisted of seventeen genotypes sharing 96.93–99.72% identity. This clade is most closely related (97.95–99.71% identity) to another *Bartonella* sequence identified in *C. horsfieldi* collected from *P. hypomelanus* in Malaysia (GenBank accession JX416256)

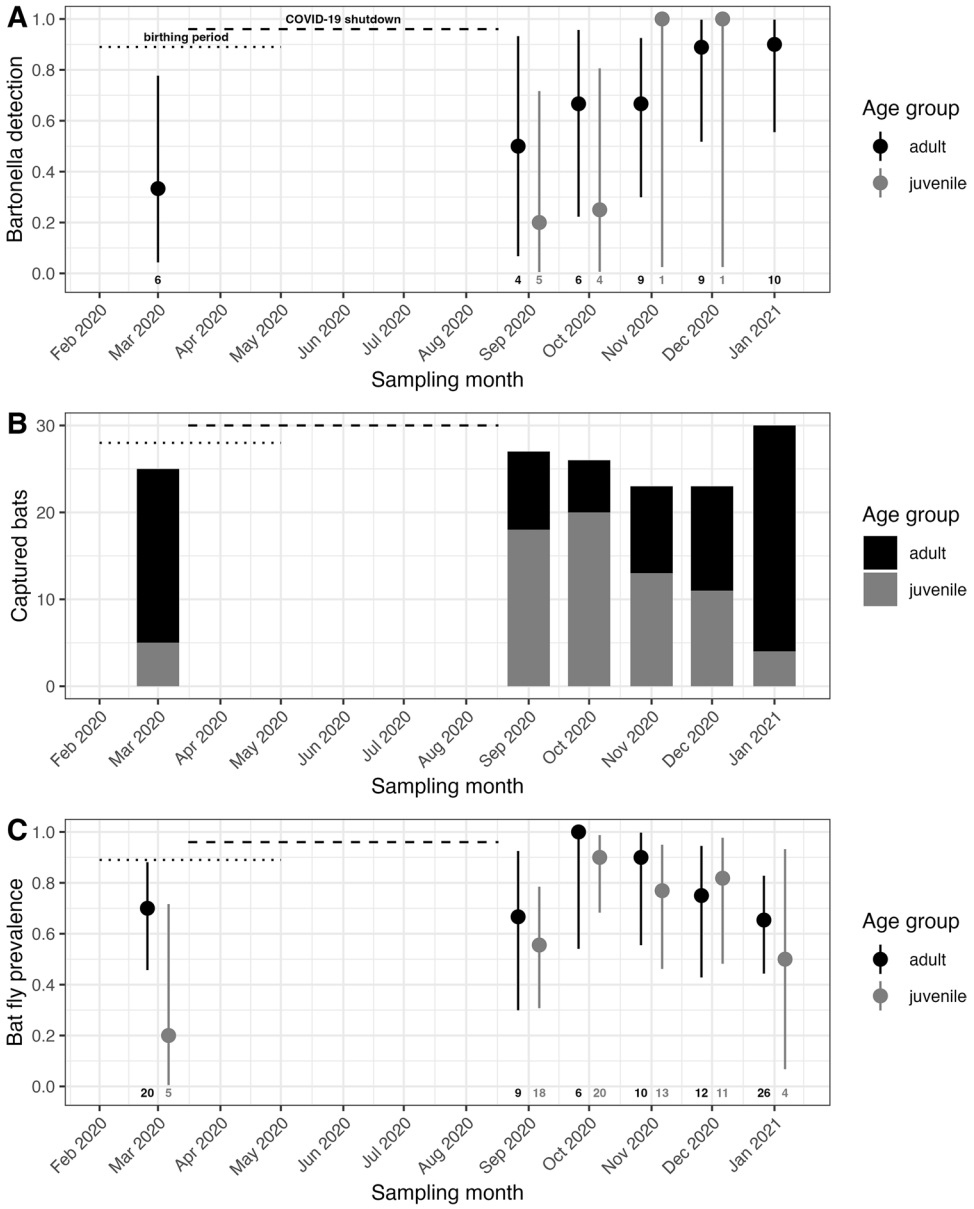
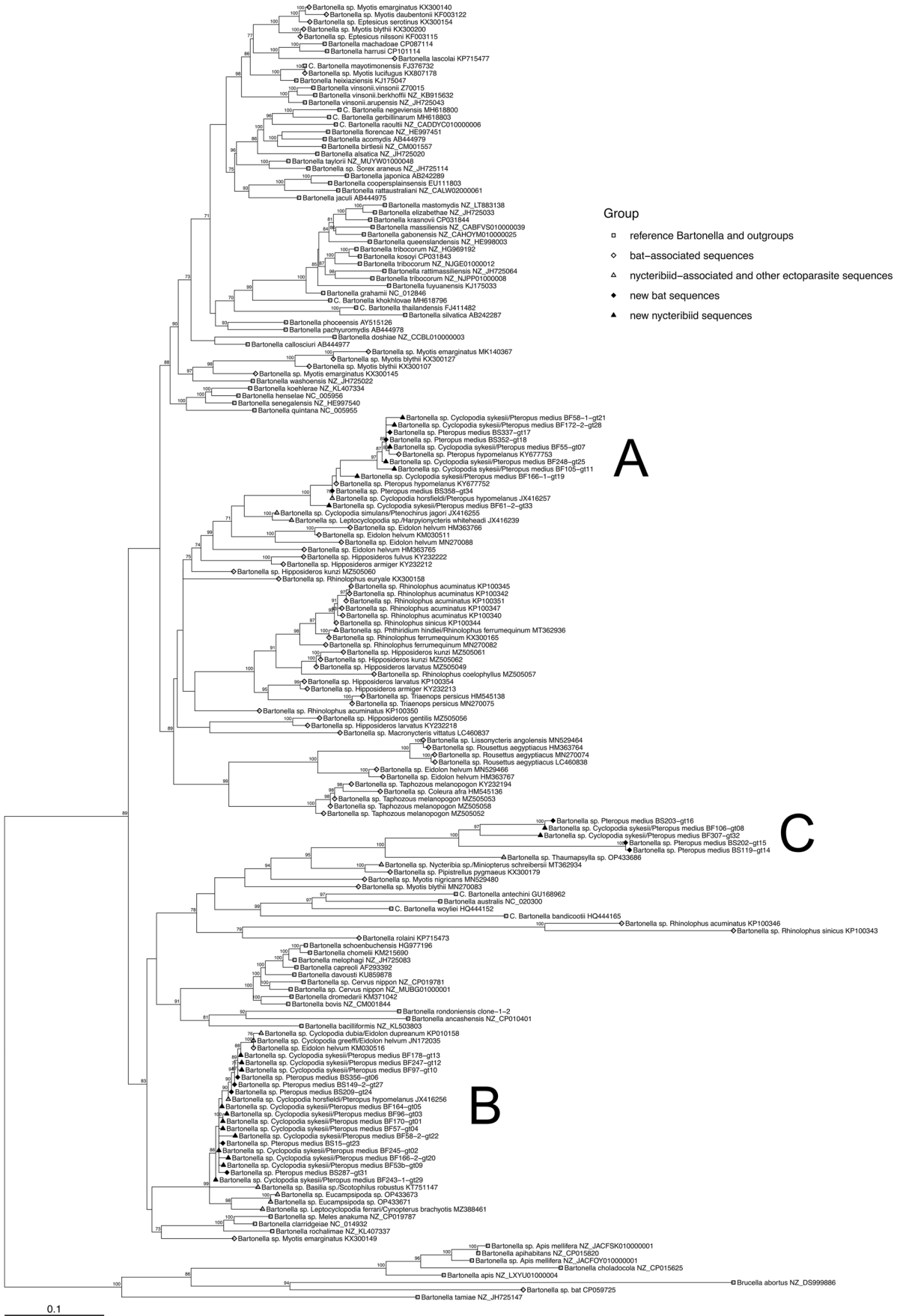


Fig. 4 The population dynamics of bats, nycteribiids, and *Bartonella* in a bat roost: Faridpur, Bangladesh. **A** The prevalence of *Bartonella* in blood spots of captured bats based on nested PCR targeting the *gltA* gene. **B** The relative counts of adult and juvenile bats captured from the roost. **C** The prevalence of nycteribiids observed on adult and juvenile bats (i.e., proportion of bats with ≥ 1 bat fly). Numbers at the bottom of **A** and **C** are the total bats captured for that age group. Confidence intervals for prevalence were estimated using the Pearson exact method

and more distantly related (93.77–8.7% identity) to sequences from *Eidolon* spp. and their associated *Cyclopodia* spp. and to sequences from other nycteribiids (GenBank accessions KM030516, JN172035, KP010158, KT751147, OP433671, OP433673, and MZ388461). The five genotypes in clade C shared a wider range of sequence identity, from 86.55% to 99.72%, potentially consisting of three distinct species. Clade C genotypes did not have any close matches in GenBank but are distantly related (< 90% identity) to other *Bartonella* sequences from vespertilionid and miniopterid bats and their bat flies and fleas (GenBank accessions OP433686, KX300179, MN529480, and MT362934). All three clades were detected in both blood spots and bat flies across the sampling period, with clade B most frequently detected (Fig. 6). Representative *gltA* sequences from the 32 *Bartonella* genotypes were stored in GenBank under accession numbers OQ584223-OQ584254.

Many of the genotypes (24/32) were unique to a single sample (Supplementary File, Tables S3 and S6), but two genotypes in clade A (gt07 and gt18) were found in three samples, and six genotypes in clade B (gt01-06) were found in at least two samples, with gt02 and gt04 occurring in 36 and 25 samples, respectively. *Bartonella* sequences in bat blood samples and bat flies from the same bat did not always match, but two pairs had sequences both in clade A and 27 pairs had sequences both in clade B. Furthermore, one pair had a matching clade A genotype (gt18), and eight pairs had matching clade B genotypes (gt02, gt03, or gt04). Five bat flies and one bat blood spot had evidence of co-infection, producing distinct clade A and clade B


Group
reference Bartonella and outgroups
bat–associated sequences
nycteribiid–associated and other ectoparasite sequences
new bat sequences
new nycteribiid sequences
A
B
C
0.1

◂**Fig. 5** Phylogenetic relationships between *Bartonella gltA* sequences. Separate groups, including new sequences detected in bats and bat flies from Bangladesh, are indicated by distinct symbols. The maximum likelihood tree was inferred using a GTR + F + I + G4 model in IQ-TREE from a 1320 bp alignment (new sequences from this study were 273-315 bp). Numbers next to nodes indicate the percent bootstrap support after 1000 replicates. Branch lengths are in units of substitutions per site

sequences following separation of Sanger reads with the Mixed Sequences Reader tool.

Discussion

Bats are important hosts of viral and bacterial pathogens [2–4], but there is little understanding of how infection varies over time or the ecological factors that influence dynamics. Pathogen shedding and spillover events may be driven by various challenges to bat health, including the presence of ectoparasites and other co-infecting pathogens [38]. To this end, we investigated the molecular prevalence and genetic diversity of *Bartonella* bacteria in *P. medius* bats and *C. sykesii* bat flies in Bangladesh. Few studies have examined *Bartonella* prevalence in pteropodid bats and nycteribiids simultaneously to understand how sequence diversity varies, none have examined temporal changes in prevalence, and none have been performed in South Asia [14, 17–21].

We detected a high prevalence and genetic diversity of *Bartonella* DNA in bat flies and bat blood in the study population in Faridpur. We also observed an increase in prevalence in bat blood from March 2020 to January 2021, coinciding with a shift in the bat age distribution and an increase in bat fly prevalence. A potential explanation for this pattern is a seasonal change in the abundance or host distribution of nycteribiids, which increased *Bartonella* transmission between bats. This pattern is supported by the high prevalence of bacteria in the sampled nycteribiids, and the discordant sequences found in paired blood and bat fly samples from individual bats, suggesting that bat flies may take blood meals from more than one bat [39]. The observation of co-infections in blood and bat flies (previously observed by [40]) during September 2020 to January 2021 (Fig. 6) also suggest that this was a period of increased transmission. Although there are very few studies on the life cycle of bat flies, nycteribiids are obligate ectoparasites that spend their entire life on their bat hosts (which may be as long as 4–6 months [39]). Females leave their hosts to deposit a prepupa on the roost substrate, but the timing of this behavior and the influence of climate or other factors on bat fly emergence and density is unknown for many species.

Nycteribiid prevalence, abundance, and intensity varies seasonally in species studied in Nigeria, Madagascar, Malaysia, and Australia [41–45], frequently exhibiting a decrease in bat fly prevalence in the bat population during months following the birth of new pups. This change may reflect preferential feeding behavior of nycteribiids on older bats and the removal of nycteribiids from pups by mothers during grooming [41]. Several studies have also noted that bat fly parasitism varies by seasonal weather conditions. The bat fly *C. greeffi* parasitizing *E. helvum* in Nigeria shows higher prevalence and intensity in the wet season than in the dry season [45] while *Eucampsipoda madagascarensis* parasitizing *Rousettus madagascariensis* shows the opposite pattern [41]. In the case of *E. madagascarensis*, the rainy season corresponds to the time shortly after the birth of new bat pups. In the case of *P. medius*, we observed higher prevalence of *C. sykesii* in the rainy season (October–November), months after pups were born and have become independent. We suspect that as juveniles aged, nycteribiid prevalence on these bats increased, resulting in the exposure of naïve bats to nycteribiids carrying *Bartonella* and an increase in transmission. The effect of rainfall may be coincidental with the demographic changes in the hosts, though more investigations of weather effects on nycteribiid survival and reproduction are warranted.

The *Bartonella* prevalence we observed in bats (64%) and bat flies (98%) was higher than previous studies on pteropodid bats and nycteribiid flies [14, 17, 19, 46], which showed PCR prevalence of 45–55% in bat blood and 42–89% in bat flies [14, 17, 19–21, 40]. While this observation might be partially explained by differing sensitivity of PCR protocols between studies, it could reflect some true difference in the burden of infection in *P. medius*. Other studies have also found evidence that male bats are more likely to be *Bartonella* positive than females and that juveniles or subadults have higher prevalence than adults [47, 48]. Our study found the opposite pattern, with higher prevalence in adults (70%) than in juveniles (36%), though this difference did not reach the level of statistical significance due to our limited sample size. These previous studies focused on New World bats, so the contrasting results between studies may reflect differences in the timing of bat fly parasitism, demographic processes, and intraspecies contact patterns across different bat-bat fly systems. Additionally, we were unable to describe when juvenile bats are first colonized by bat flies and infected with *Bartonella*. Due to national lockdowns due to COVID-19 in 2020, our field team could not sample bats in April and May when pups are born into the colony. The juvenile bats sampled starting in September 2020 were a mixture of bats born in 2020 and the previous year. Due to size overlap between juveniles and adults, we cannot reliably distinguish these juvenile cohorts without analyzing tooth cementum [49, 50]. Additional longitudinal studies of *Bartonella* in fruit bats will be necessary to gather more detailed knowledge on the age-specific force of infection for

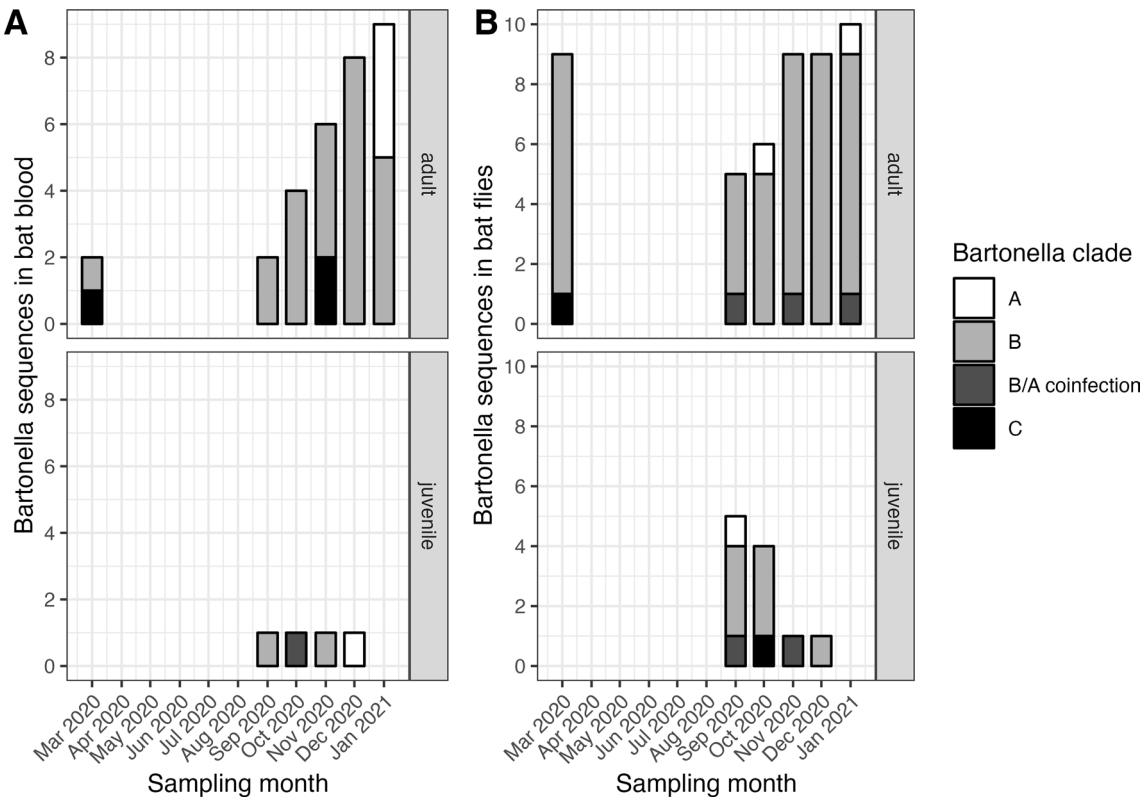


Fig. 6 Clades of *Bartonella* sequenced in bat blood (**A**) and nycteribiids (**B**) from *P. medius* in a bat roost in Faridpur, Bangladesh. Separate panels depict the mixture of clades in the separate age groups of captured bats

Bartonella bacteria and compare that to patterns observed for viruses [22].

To our knowledge, this is the first published record of *Cyclopodia sykesii* from Bangladesh, although there are records from the neighboring Indian states of Assam and West Bengal [23, 37, 51]. There are few recent records of this arthropod, except a study of *C. horsfieldi* in flying foxes that potentially detected a male *C. sykesii* (morphologically indistinguishable from *C. horsfieldi*) from *Pteropus vampyrus* in Malaysia [52]. A recent report from Odisha, India, described the morphology of *C. sykesii* from *P. medius*, the first reporting of this species in that region in 100 years [51]. The molecular marker we used to genotype nycteribiids (16S rRNA) was used instead of the usual *COI* barcode because the 16S rRNA PCR protocol has more amplification success in our experience (C. McKee, unpublished data). The phylogenetic relationship between *C. sykesii* and *C. greeffi* in the 16S rRNA tree (Fig. 4) is likely an artifact due to a lack of 16S rRNA sequences from other *Cyclopodia* species on GenBank. Further comparisons of *COI* and other genetic markers from *C. sykesii* would refine our understanding of its evolutionary relationship within the *Cyclopodia* genus.

Our estimates of *Bartonella* prevalence in bats and bat flies may not be representative of the entire population in the Faridpur roost because we only tested a subset of bats that had paired blood spots and bat flies, although the majority of captured bats had bat flies. Additionally, we did not quantify the total burden of bat flies and other ectoparasites. Ectoparasite burden could affect prevalence in bats, although individual bat's ectoparasite load at the time of sampling has not been consistently correlated with infection status in the few studies that have investigated this hypothesis [47, 48]. Examining blood from bats without ectoparasites and additional sites with varying abundance of bat flies and other ectoparasites will be necessary to understand the drivers of high *Bartonella* prevalence in *P. medius* and the role that bat flies play as vectors of bartonellae. The transmission route for *Bartonella* spp. to bats from their ectoparasites is largely unknown and could involve eating vectors, inhaling or consuming infectious arthropod feces, by bite of infected vectors, or other routes. We also know little about the timing of reproduction and the population cycles of nycteribiids, especially *Cyclopodia* species. More detailed research on the life cycle of nycteribiids could clarify some of the observed dynamics of *Bartonella* transmission in bats.

In addition, our characterization of *Bartonella* diversity only targeted variation in a single genetic marker (*gltA*) and could only measure the presence of *Bartonella* DNA, not viable bacteria. Previous studies have cultured bartonellae from pteropodid blood and nycteribiids and tend

to show lower prevalence using this method (10–26%) compared to PCR [17, 21, 40, 53], partly due to slow growth of the bacteria, its tendency to be overgrown by other bacteria and fungi, and low bacteremia. Nevertheless, it is likely that some of the PCR-positive bats and bat flies in our study have cultivable bartonellae. Attempts to culture bartonellae from *P. medius* or bat flies followed by genome sequencing or multi-locus sequence typing could clarify the phylogenetic relationships of the *Bartonella* clades detected in this study.

Despite these caveats, our study demonstrated that *P. medius* and their ectoparasitic bat flies have a high prevalence and diversity of *Bartonella* bacteria. This pilot study also showed that *Bartonella* prevalence varies over time, potentially in response to seasonal reproductive cycles in bats and bat flies. Additional investigations in this system, with efforts to understand population cycles of bats and bat flies in different roosts, would be beneficial for understanding *Bartonella* transmission and the consistency of seasonal dynamics. The high prevalence of *Bartonella* in *P. medius* and *C. sykesii* could also prove fruitful for additional studies that explore other ecological aspects of this host-pathogen system. For example, *P. medius* populations in Faridpur are genetically distinct from those in eastern Bangladesh due to limited migration and gene flow [22, 54]. Exploration of the population genetics of bat flies may show concordant or discordant population structure compared to bat hosts, which could reveal patterns of bat movement within Bangladesh [52, 55]. This system could also be used to explore the relationships among *Bartonella* infection, ectoparasite load, viral infection, and bat health. Whether *Bartonella* infection could be a result of poor health, a cause of poor health, or have no effect on bat health, is still to be determined. Studies investigating bacteria and host-ectoparasite relationships in bats will bolster our ability to characterize bat health and physiology and may help identify drivers of viral spillover and cross-species transmission.

The public health implications of these results are difficult to assess, though the potential for cross-species *Bartonella* transmission from bats to humans has been demonstrated in several investigations [14, 56]. With the close proximity of *P. medius* to urban areas, people may come into contact with bats on the ground or in fruit trees, possibly leading to bites or scratches [57]. Hunting of *P. medius* also occurs in Bangladesh, so there may be additional exposures during capture and slaughter of animals [58]. The risk of nycteribiids biting humans is not clear, though other related louse flies and bat flies will occasionally bite humans [59, 60]. Additional investigations on the exposure of humans to bat-borne *Bartonella* and the interactions between humans and bats in Bangladesh would help to assess potential interventions that support bat conservation and protect human health.

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Code Availability The R code used for performing statistical tests and generating figures is available from the corresponding author on reasonable request.

Author Contribution A.I. collected the samples, A.C.F. and W.K.R. performed the laboratory work, and A.C.F. and C.D.M. performed the data analysis. A.C.F. and C.D.M. wrote the main manuscript text, C.D.M. prepared all figures, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files (Tables S1-S6). Sequences generated as part of this study are available on GenBank with accession numbers OQ401037, OQ401038, and OQ584223-OQ584254.

Declarations

Ethics Approval This study was conducted under protocol PR#2006-012 approved by the Research Review Committee and Animal Experimentation Ethical Committee of icddr,b (formerly the International Centre for Diarrhoeal Disease Research, Bangladesh) with permission from the Forest Department, Government of Bangladesh.

Informed Consent No human subjects were included as part of this study.

Competing Interests The authors have no competing interests to declare that are relevant to the content of this article.

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