

# Chlamydiales and hemotropic mycoplasma in captive and free-living bats

**Janine Fritschi**

University of Zurich

**Hanna Marti**

University of Zurich

**Helena M.B. Seth-Smith**

Universität Zurich Institut für Volkswirtschaftslehre

**Sébastien Aeby**

University of Lausanne

**Gilbert Greub**

University of Lausanne

**Marina L. Meli**

Universitäts-Kinderspital Zurich

**Regina Hofmann-Lehmann**

University of Zurich

**Kristin Mühldorfer**

Leibniz Institute for Zoo and Wildlife Research

**Nadine Stokar-Regenscheit**

Universität Bern

**Danja Wiederkehr**

Bern University of Applied Sciences

**Paola Pilo**

Universität Bern

**Peggy Van Den Broek**

Stiftung Papiliorama

**Nicole Borel** (✉ [nicole.borel@uzh.ch](mailto:nicole.borel@uzh.ch))

University of Zurich

---

## Research article

**Keywords:** Chlamydiales, hemotropic mycoplasma, hemoplasmas, bats, DNA, qPCR

**Posted Date:** January 28th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.22015/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

---

**Version of Record:** A version of this preprint was published on June 26th, 2020. See the published version at <https://doi.org/10.1186/s12866-020-01872-x>.

## Abstract

**Background:** Bats are hosts for a variety of microorganisms, however, little is known about the presence of *Chlamydiales* and hemotropic mycoplasma. This study investigated 475 free-living and captive bats from Switzerland, Germany and Costa Rica for the occurrence of *Chlamydiales* and hemotropic mycoplasma.

**Results:** Screening for *Chlamydiales* was performed using a *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene and a pan-*Chlamydiales* PCR targeting the 16S rRNA gene resulting in a total prevalence of 31.4%. For sequencing, a PCR with the specifically designed inner primers panFseq and panRseq was performed, and criteria published by Pillonel et al. were used to classify the 19 obtained sequences, resulting in the formation of two groups. Groups one and two shared sequence identities to *Chlamydiaceae* and to *Chlamydia*-like organisms, including *Rhabdochlamydiaceae* and unclassified *Chlamydiales* from environmental samples, respectively.

Analysis for the presence of hemotropic mycoplasma was performed using a universal SYBR Green hemoplasma screening real-time PCR targeting the 16S rRNA gene, real-time PCRs specific for *M. haemofelis*-like and '*Candidatus M. haemominutum*'-like organisms and two conventional PCRs targeting an 871-bp and 1030-bp region of the 16S rRNA gene resulting in a total prevalence of 0.7%. Sequencing and phylogenetic analysis of the 871-bp and 1030-bp region of the 16S rRNA gene were used to classify positive specimens and infer their phylogenetic relationships. Three sequences with identities to other unidentified mycoplasma found in vampire bats and Chilean bats were obtained.

**Conclusions:** Bats can harbor *Chlamydiales* and hemotropic mycoplasma and the newly described sequences in this study indicate that the diversity of these bacteria in bats is much larger than thought before. Both, *Chlamydiales* and hemotropic mycoplasmas are not restricted to certain bat species or countries and free-living as well as captive bats can be colonized. In conclusion, bats represent another potential host or vector for novel, previously unidentified, *Chlamydiales* and hemotropic mycoplasma.

## Background

Bats are of increasing interest as potential reservoirs and vectors of pathogens. They possess unique characteristics among mammals, such as the ability to fly. Their extensive mobility, combined with their roost plasticity, nesting behavior and broad food range allows transport of pathogens to many different animal species in various locations (1). It has been shown that bats are hosts for a multitude of different microorganisms that include viruses, bacteria, parasites and fungi. Several of these infectious agents are common to humans and domestic animals. For some bacterial species, the pathogenic potential has been confirmed for bats, but the knowledge regarding the impact of such microorganisms on bat hosts is limited. In addition, knowledge on the natural microbiota of bats is scarce (2).

*Chlamydiae* are highly successful animal and human pathogens. Their taxonomic structure is composed of the order *Chlamydiales*, which consists of the nine families *Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae*, *Rhabdochlamydiaceae*, *Criblamydiaceae*, *Piscichlamydiaceae*, *Clavichlamydiaceae* and *Parilichlamydiaceae*, collectively referred to as *Chlamydia*-like organisms, and the *Chlamydiaceae* (3–5). *Chlamydiae* are obligate intracellular bacteria with a biphasic developmental cycle. This cycle is characterized by the infectious but metabolically less active elementary body, which infects susceptible host cells, and the intracellular reticulate body, which undergoes binary fission (6). As an inclusion fills with progeny, the reticulate bodies condense back into elementary bodies and are released by host cell rupture or by fusion of the inclusion membrane with the host cell plasma membrane. *Chlamydiae* are disseminated by aerosol or contact, requiring no alternative vector (4).

In 2005 and 2015, two novel *Chlamydia*-like organisms, *Waddlia malaysiensis* and *Waddlia cocoyoc* were detected in fruit bats in Malaysia and Mexico, respectively (7, 8). In 2016, members of the order *Chlamydiales* were detected in the fecal bacterial microbiota of Daubenton's bats in Finland (9).

Hemotropic mycoplasma (hemoplasmas) are considered to be emerging or re-emerging zoonotic pathogens, classified taxonomically in the order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Mycoplasma*. They are small, pleomorphic bacteria without a cell wall and parasitize red blood cells (10, 11). The transmission routes of hemoplasmas are not yet fully understood, but they are thought to be transmitted through blood, saliva and possibly also arthropods (12–14, 10). Hemoplasmas are able to cause acute infectious anemia or various chronic diseases in farm animals (15, 16), wild animals (17, 18), pets (14) and humans (19). The extent of clinical manifestations ranges from asymptomatic to life-threatening (10).

From 2014 to 2017, four studies to determine the prevalence of hemoplasmas in various bat species were conducted (20–23). The prevalence ranged from 18.5% in Brazil to 96.8% in Spain and sequences indicating new species or new genotypes were identified (20–23).

The objective of this study was to investigate the occurrence and phylogenetic positioning of *Chlamydiae* and hemotropic mycoplasma species in 475 captive and free-living bats from six families and 28 species from Switzerland, Germany and Costa Rica (Table 1).

## Results

### PCR and sequencing results for *Chlamydiales*

A total of 166/1021 DNA samples (16.3%) originating from 149/475 bats (31.4%) were positive for *Chlamydiales* DNA by real-time PCR. Of these 166 samples, 15 samples had a Ct value below 35.0 and were therefore sequenced, resulting in 21 sequences that were readable from both directions (Table 2). A BLAST analysis of these sequences revealed two groups:

Group one contained ten sequences from seven free-living bats originating from Switzerland and Germany that belong to the *Chlamydiaceae*-family according to the classification scheme by Pillonel et al. (24). Within this group, the best BLAST hits for the sequences obtained by the 16S-IGF/IGR-PCR were with sequences from *Chlamydia pecorum* (strain B0-Maeda [GenBank accession number AB001775.1]) showing a low sequence identity of 93.3 to 94.1%; and from *Chlamydia trachomatis* (strain SQ24 [GenBank accession number CP017733.1]) with a sequence identity of 93.2%. Thus, these sequences are from a new species-level lineage within the *Chlamydiaceae* that we propose to name "*Candidatus Chlamydia myotis*" since one of the sequence was retrieved from a *Myotis myotis* bat (a free-living species from Switzerland).

The best BLAST hits of the sequences obtained by the 16S-pan-PCR were with sequences from uncultured *Chlamydiales* bacterium clone 1778 (GenBank accession number KU664271.1) with a sequence identity of 98.9 to 99.5%, from *Chlamydiales* bacterium isolate CL gt1 (GenBank accession number MF620054.1) with a sequence identity of 97.5% and from *Chlamydia* sp. (S15-834C isolate [GenBank accession number LS992154.1]) with a sequence identity of 95.8%.

Group two contained eleven sequences from four captive and seven free-living bats originating from Switzerland and Germany; they are closely related to sequences from *Chlamydia*-like organisms. The best BLAST hits were with sequences from uncultured *Chlamydiales* and bacterium clones with a sequence identity of 92.4 to 100% and from the *Rhabdochlamydiaceae* bacterium isolate P gt1 (GenBank accession number MF620051.1) with a sequence identity of 96.6%, thus representing a new species-level lineage within the *Rhabdochlamydia* genus, that we propose to name "*Candidatus Rhabdochlamydia rhogeessa*" according to the genus name of the free-living bat from Costa-Rica.

The phylogenetic trees (Figure 1) constructed from the 16S rRNA and 23S rRNA gene sequences of group one and the *Rhabdochlamydiaceae*-like sequence confirmed that these sequences fall together in two novel species-level lineages (Figure 1A) and are closely related to previously published sequences from Finnish bat samples (Figure 1B, C) (9).

### PCR and sequencing results for hemotropic mycoplasma

A total of 15/475 DNA samples (3.2%) originating from 15/462 bats (3.3%) tested positive or questionably positive for hemotropic mycoplasmal DNA by both or at least one of the two real-time PCRs used for screening. All 15 samples were also positive by conventional PCR and were sent for sequencing, which resulted in 12 sequences that were readable in both directions (Table 2). BLAST analysis revealed three sequences that were closely related to uncultured *Mycoplasma* sp. (clone 20180131LOC1.16 and clone D159 [GenBank accession numbers MK295631.1 and KY932722.1]) with a sequence identity of 96.3 to 98.6% (Table 2). The phylogenetic tree constructed from these 16S rRNA gene sequences (Figure 1D) showed that they are closely related to previously published sequences from vampire bat samples (23) and sequences from Chilean bat samples (25).

The remaining nine sequences were closely related to Proteobacteria by BLAST analysis (Table 2).

## Discussion

Bats are known to be important vectors and reservoirs for a multitude of different microorganisms such as lyssaviruses, henipaviruses (2), *Leptospira* sp. (26) and *Bartonella* spp. (27), but the knowledge regarding their potential role as carriers of other clinically significant

pathogens or less common bacteria is limited. To date, the only *Chlamydiales* identified in bats are two *Chlamydia*-like organisms: *Waddlia malaysiensis* (7) and *Waddlia cocoyoc* (8), isolated from fruit bats in Malaysia and Mexico, respectively, and members of the order *Chlamydiales*, isolated from a common bat species (*Myotis daubentonii*) in Finland (9).

### PCR and sequencing results for *Chlamydiales*

BLAST analysis of the sequences obtained in the present study resulted in the formation of two groups: *Chlamydiaceae*-likes and *Chlamydiales*-like. Separation in these groups had been previously described in the study from Hokynar et al (9). Group one shared sequence similarities to *Chlamydiaceae* and group two to *Chlamydia*-like organisms, but three samples yielded sequences, which were represented in both groups. The latter finding could be attributed to the fact that a precise classification according to the scheme from Pillonel et al. (24) is not possible based on short amplicons as retrieved in this study and also because only 16S rRNA and not the housekeeping genes described in Pillonel et al. (2015) was sequenced. The phylogenetic trees showed that five samples from group one are closely related to the samples from Hokynar et al. and formed a novel clade next to already known members of the *Chlamydiaceae* family. These five samples originated from free-living bats from Switzerland and Germany belonging to the bat species *Myotis myotis*, *Pipistrellus* sp., *Pipistrellus pipistrellus*, *Nyctalus noctula* and *Eptesicus serotinus*. Therefore, it can be said that this clade first discovered in Finland occurs also in other parts of Europe and colonizes various species of free-living insectivorous bats. In the present study, these *Chlamydiae* were detected directly in the inner organs; therefore, these bats might actually act as a host, meaning that the *Chlamydiae* are not merely prey-borne, as hypothesized by Hokynar et al. The latter study only investigated bat faeces and insect material, inner organs were not available. Although the present study indicates the presence of chlamydial DNA in inner organs, such as the intestine, it could not be assessed whether replicating chlamydial organisms were present; Detection of replication would have required the attempt of isolation, which was hampered by the unavailability of fresh frozen tissue material. Chlamydial DNA might represent colonization and/or real infection. If the latter holds true, this would suggest that the different chlamydial species documented in bats might have a pathogenic role for the bats or that these bats act as vectors.

In a study by Hornok et al. (28), 196 individual and 25 pooled fecal samples collected from 19 bat species from Hungary and the Netherlands were investigated for the presence of chlamydial DNA; they all tested negative indicating that the prevalence of *Chlamydiae* in bats is highly variable in studies from neighboring countries. This observation suggests that, although this new *chlamydiae* clade may occur in some parts of Europe, colonization or infection of bats is influenced by factors that remain to be defined. Methodological differences may play a part of these factors.

Also a *Rhabdochlamydiaceae*-like sequence was identified in a sample taken from a free-living bat from Costa Rica, which formed another novel clade with bat samples from Hokynar et al. (9) in the phylogenetic tree. The remaining bat samples, similar to chlamydial sequences from environmental samples retrieved from the NCBI database, originated from free-living and captive bats from Switzerland and Germany. *Chlamydia*-like organisms are commonly present in water and inside amoebae (29-31), and have also been observed in ticks (32, 33) and fleas (34). Thus, it is difficult to guess, which of these chlamydial putative vectors (amoebae or ticks) is the more likely to play as reservoir and vector.

In previous studies from Chua et al. (7) and Piérle et al. (8), two novel *Chlamydia*-like organisms belonging to the *Waddliaceae* family and named *Waddlia malaysiensis* and *Waddlia cocoyoc* were isolated from fruit bats from Malaysia and the municipality Cocoyoc in Mexico. These regions have a tropical climate, whereas a temperate climate predominates in Switzerland and Germany. In this study, bats from a tropical climate (Costa Rica) were analysed as well, but compared to the two other studies (n=206 and n=38) our sample size from Costa Rica (n=17) was small. In the bats investigated in this study, no *Waddliaceae* could be detected. *Waddlia malaysiensis* was first detected in urine samples from bats (7), which had not been investigated in the current study. *Waddlia cocoyoc* was detected in DNA from the skin and in infected Vero and BHK 21 cells, but also caused severe lesions in lungs and spleen (8). Therefore, it can be assumed that bacterial DNA would have been detected in these two organs if bacteria were present. The two isolates from Mexico and Malaysia might be bound to the regions and/or the climate. Hence, bacterial DNA was undetectable in the European bat samples in this investigation and are unlikely to get detected in the samples from other European studies.

In this study as well as in the studies from Hokynar et al. and Chua et al., whole genome sequencing was not performed; only short fragments of the 16S rRNA and 23S rRNA genes were amplified, as many of the samples in the present study were only available as FFPE samples because the bats had died in the wild and were subsequently collected and sampled, therefore preventing detailed classification.

### PCR and sequencing results for *Mycoplasmatales*

BLAST analysis of the sequences obtained in the current study resulted in the identification of three sequences that were closely related to uncultured *Mycoplasma* sp. and nine sequences that were closely related to *Proteobacteria*. The amplification of these *Proteobacteria*-like sequences is most likely the result of a cross-reaction or unspecific primer binding.

The three sequences related to uncultured *Mycoplasma* sp. originated from free-living bats from Germany and Costa Rica belonging to the bat species *Nyctalus noctula*, *Vespertilio murinus* and *Glossophaga commissarisi*. Consequently, different species from the bat families *Vespertilionidae* and *Phyllostomidae* can harbor hemotropic mycoplasma. The occurrence of mycoplasma could be only confirmed in free-living bats, which is in agreement with the presumed transmission pathways of hemotropic mycoplasma through blood, saliva and arthropods (12–14, 10). The captive bats were isolated from the environment and thus arthropod contact was at least partially excluded or reduced. Moreover, none of them were sanguivory bats and therefore do not bite other animals living in the enclosure. Consequently, transmission by blood, saliva and arthropods is very unlikely in captive bats.

The phylogenetic tree shows a close relationship between the sequences of the German bats among each other and a sequence from a Chilean bat (25). The sequences from Costa Rican bats, on the other hand, were more closely related to sequences of vampire bats from Peru and Belize (23). Vampire bats and the Commissaris's long-tongued bat (*Glossophaga commissarisi*) belong to the family of leaf-nosed bats and Peru, Belize and Costa Rica are geographically close to each other strengthening the high degree of relationship in the phylogenetic tree.

In other studies by Ikeda et al. (20), Mascarelli et al. (21), Millan et al. (22) and Volokhov et al. (23), a much higher mycoplasmal prevalence (18.5% to 97%) was found than in the present study (0.7%), despite partially similar numbers of samples. Only in the study by Hornok et al. (28), a similar prevalence was reported (1.8%). Millan et al. suggested that subclinical infections with mycoplasma in bats are common, which could not be confirmed in the present study. This may be related to distinct climatic conditions and thus different arthropod activity, which are discussed as a transmission route. In all studies, only fragments of the 16S rRNA gene were amplified, sequenced and then combined into contigs where possible to optimize the chances of getting a 16S rRNA gene sequence to analyse. Whole genome sequencing was not performed. Due to high blood content in the spleen, and samples only available as FFPE samples, methods for DNA analysis in the present study were limited. Therefore, a more detailed description of the analysed sequences and a more precise classification into the mycoplasmal taxonomy were not possible.

According to the classification of mammals, bats belong to the superorder of the *Laurasiatheria* and therefore are more closely related to carnivores, even-toed and odd-toed ungulates, than to rodents and primates, which belong to the superorder of the *Euarchontoglires*. While the relationship of other hemotropic mycoplasma represented in the phylogenetic tree (Figure 1D) roughly corresponds to that of the mammalian classification, *Mycoplasma* of bats are the only ones to classify quite differently. This is likely due to the high resistance of bats to pathogenic microbes, somehow similar to some rodents (mice, rats). Moreover, this suggests that bats does not get infected by exposure to meat, but rather by exposure to water (and free-living amoebae) as well as to ectoparasites colonizing bats (such as *Spinturnix*). The latter hypothesis is supported for *Chlamydia*-like organisms by the recent work of Thiévent et al. that demonstrated the occurrence of *Chlamydia*-related bacteria in several *Spinturnix* species (35).

In conclusion, bats can harbor *Chlamydiales* and hemotropic mycoplasma and the newly described sequences in this study indicate that the diversity of these bacteria in bats is much larger than thought before. Both, *Chlamydiales* and hemotropic mycoplasmas are not restricted to certain bat species or countries and free-living as well as captive bats can be colonized.

## Conclusions

Bats are carriers of several pathogenic viruses and seem to be reservoir hosts for a variety of bacteria. Chlamydiaceae, Chlamydia-like organisms and hemotropic mycoplasma have been identified in humans and various animal species but only a few studies on their presence in bats have been performed. In this study, the occurrence of chlamydial and mycoplasmal DNA in free-living and captive bats from Switzerland, Germany and Costa Rica was assessed. Chlamydial and mycoplasmal sequences similar to sequences obtained from bats and their prey investigated in other studies and from environmental samples were identified, suggesting bats are hosts and/or vectors for novel, previously unidentified members of the Chlamydiales order and hemotropic mycoplasma.

## Methods

### Bat sampling and DNA extraction

A total number of 475 bats belonging to six bat families and 28 bat species were sampled (Table 1). Lungs, liver, intestines and spleen from 89 captive bats from Switzerland, 28 captive and 55 free-living bats from Germany, and 17 free-living bats from Costa Rica were investigated. Thereof, bat samples from Germany and Costa Rica were obtained from the Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany and samples from Switzerland were obtained from the Institute of Animal Pathology, Vetsuisse-Faculty, University of Bern, Switzerland, from the Stiftung Papiliorama, Kerzers, Switzerland and from Dr. Danja Wiederkehr.

DNA extraction from bat samples from Switzerland was performed using the DNeasy Blood and Tissue Kit #69506 (Qiagen, Hilden, Germany) following manufacturer's instructions, whereas DNA extraction of the samples from Germany and Costa Rica was performed using either the NucleoSpin® DNA RapidLyse (lung, liver, spleen) or the NucleoSpin® Tissue Kit (intestine) (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Additionally, inner organs including lungs, liver, intestines and spleen, from 285 free-living bats from Switzerland were obtained either as FFPE blocks or fixed in 4% formalin. The DNA of the FFPE blocks was extracted directly, while the organs fixed in 4% formalin were first embedded in paraffin according to routine procedures. The DNA extraction was then performed using the QIAamp DNA FFPE Tissue Kit #56404 (Qiagen) following manufacturer's instructions. DNA quantity and quality of all samples was evaluated with the Nanodrop-1000 (Witec AG, Luzern, Switzerland).

### PCR analysis for *Chlamydiales* DNA

A total of 1021 DNA samples was screened for the presence of *Chlamydiales* DNA using two different real-time PCRs targeting sequentially the 23S rRNA gene (*Chlamydiaceae* family-specific) and the 16S rRNA gene (pan-*Chlamydiales* order-specific).

The *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene (Chlam23S-qPCR) (36) used primers Ch23S-F, Ch23S-R and probe Ch23S-p (Microsynth, Balgach, Switzerland) described by Ehricht et al. (37). The internal amplification control eGFP amplified with primers eGFP-1-F, eGFP-10-R and probe eGFP-Hex (Microsynth) was added to each reaction (38). The PCR was conducted on a Thermocycler 7500 Fast ABI (Thermo Fisher Scientific). All samples were tested in duplicate and samples with a cycle threshold of <38 in duplicate PCR reactions were considered positive. Quantitation was performed using 10-fold dilutions ( $10^7$  copies to 10 copies/ $\mu$ L) of the *Chlamydia abortus* genomic DNA positive control (standard curve).

Samples positive in the *Chlamydiaceae* family-specific real-time PCR were then further analysed using three different conventional PCR protocols targeting partial sequences of the 16S or 23S rRNA gene. The conventional PCR targeting a 278-bp fragment of *Chlamydiales* 16S rRNA gene (16S-IGF/IGR-PCR) was performed using primers 16S-IGF and 16S-IGR (39,40) (Microsynth) modified from Everett et al. (4). Primers 16S-panCh-F and 16S-panCh-R (Microsynth) were applied for the conventional PCR targeting a 200-bp fragment of the *Chlamydiales* 16S rRNA gene (16S-pan-PCR) (29) and the conventional PCR targeting a 700 bp fragment of *Chlamydiales* 23S rRNA gene (23SIG-PCR) (32, 4, 36) included primers U23-F and 23SIG-R (Microsynth). For all three conventional PCRs, cycling was performed on a Biometra TRIO Thermal Cycler (Analytik Jena AG, Jena, Germany) and PCR products were analysed by gel-electrophoresis on a 1.5% agarose gel.

For the pan-*Chlamydiales* real-time PCR targeting a partial sequence of the 16S rRNA-encoding gene (16S-pan-qPCR) (41), primers 16S-panCh-F, 16S-panCh-R and probe 16S-panCh (Eurogentec, Seraing, Belgium) were applied in a StepOne Plus real-time PCR system (Thermo Fisher Scientific). All samples were tested in duplicate, and if a single replicate was positive (Ct  $\leq$  37), the corresponding sample was considered positive. Quantification was performed using a 10-fold-dilution of a plasmid control tested in duplicate, constructed with the sequence of interest based on the *Parachlamydia acanthamoebae* 16S rRNA encoding gene, cloned with the TOPO TA Cloning Kit for Subcloning with One Shot TOP10 chemically competent *E. coli* cells (Thermo Fisher Scientific). Molecular-biology-grade water was used as a negative control in all PCR reactions.

All PCR primers and probes, the targeted genes and amplicon sizes used in this study are summarized in Table 3. All reaction mix compositions and cycling conditions are shown in Table S1.

### PCR analysis for hemotropic mycoplasma DNA

All spleen and FFPE block samples (n = 475) from 462 bats were screened for the presence of hemoplasma DNA using a universal hemotropic mycoplasma-specific SYBR Green real-time PCR (Hemoplasma SYBR Green qPCR) targeting the 16S rRNA gene, including primers Mhae\_sybr.359f, Mcocc\_sybrF, Mhae\_sybr.432r and Cmhae\_Sybr.493r (Microsynth) (42). Samples within the same melting temperature range as the positive controls were considered positive.

Samples positive by SYBR Green real-time PCR were then further analysed by using real-time PCRs specific for *M. haemofelis*-like and '*Candidatus M. haemominutum*'-like organisms and two conventional PCRs targeting an 871-bp and 1030-bp region of the 16S rRNA gene.

The *M. haemofelis*-like-specific real-time PCR (Mhf-like qPCR) targeting a 114-bp fragment of the 16S rRNA gene was performed using primers Group\_Mhf\_fwd and Group\_Mhf\_rev and probe Group\_Mhf\_probe (Microsynth) (43).

The '*Candidatus M. haemominutum*'-like-specific real-time PCR (CMhm-like qPCR) targeting a 139-bp fragment of the 16S rRNA gene included primers Group\_CMhm\_fwd and Group\_CMhm\_rev and probe Group\_CMhm-probe (Microsynth) (43).

All three real-time PCRs were conducted on a Thermocycler 7500-Fast ABI. Molecular-biology-grade water was used as a negative PCR control and DNA of *Mycoplasma haemofelis*, '*Candidatus Mycoplasma haemominutum*', '*Candidatus Mycoplasma turicensis*' and '*Candidatus Mycoplasma haematoparvum*' was used as a positive PCR control.

The conventional PCR targeting an 871-bp fragment of Hemoplasma 16S rRNA gene (HemMycop41/938-PCR) was performed using primers HemMycop16S-41s and HemMycop16S-938as (Microsynth). Primers HemMycop16S-322s and HemMycop16S-1420as (Microsynth) were applied for the conventional PCR targeting a 1030-bp fragment of the Hemoplasma 16S rRNA gene (HemMycop322/1420-PCR) (21). Molecular-biology-grade water was used as a negative PCR control and DNA from both *Mycoplasma wenyonii* and *Mycoplasma haemocanis* were used as positive PCR controls. Cycling was performed on a Biometra T-personal Thermal Cycler (Biolabo Scientific Instruments, Châtel-Saint-Denis, Switzerland) and PCR products were analysed by gel-electrophoresis on a 1.5% agarose gel.

All PCR primers and probes, the targeted genes and amplicon sizes used in this study are summarized in Table 3. All reaction mix compositions and cycling conditions are shown in Table S1.

### **Sequencing and analysing of *Chlamydiales*- and Hemoplasma-positive PCR products**

For sequencing, amplicons of samples positive by conventional PCRs 16S-IGF/IGR-PCR, 16S-pan-PCR, 23SIG-PCR, HemMycop41/938-PCR or HemMycop322/1420-PCR were purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific) or the GeneJET Gel Extraction Kit (ThermoFisher Scientific) according to manufacturer's instructions. The forward and reverse strands of the PCR products were sequenced using the respective primers of the positive PCR reaction. Microsynth performed all sequencing reactions using Sanger sequencing.

Amplicons of samples positive by 16S-pan-qPCR with a Ct value  $\leq$  35.0 were a) either purified using an MSB Spin PCRapace kit (Invitex, Berlin, Germany) with a subsequent PCR reaction using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) (41) or b) purified and sequenced by Microsynth using specifically designed inner primers panFseq and panRseq.

Sequences were compared to known sequences in the NCBI database by BLAST analysis and phylogenetic analyses were performed using muscle in Seaview (44) with manual correction where necessary to create alignments, and using PhyML in Seaview with default parameters to create phylogenetic trees.

### **Accession numbers**

The sequences obtained were deposited in the GenBank under accession numbers LR699022.1, LR699021.1, LR699020.1, LR584972.1, LR584971.1, LR584970.1; LR584969.1, LR584968.1, LR584967.1, LR584966.1, LR584965.1, released under the project 546195: <https://www.ncbi.nlm.nih.gov/nuccore?term=546195%5BBioProject%5D>

## **Declarations**

### **Ethics approval and consent to participate**

We obtained written informed consent to use bat samples from the Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, from the Institute of Animal Pathology, Vetsuisse-Faculty, University of Bern, Switzerland, from the Stiftung Papiliorama, Kerzers, Switzerland and from Dr. Danja Wiederkehr.

### **Consent of publication**

Not applicable.

### Availability of data and material

The sequences obtained in this study were deposited in the GenBank under accession numbers LR699022.1, LR699021.1, LR699020.1, LR584972.1, LR584971.1, LR584970.1; LR584969.1, LR584968.1, LR584967.1, LR584966.1, LR584965.1, released under the project 546195: <https://www.ncbi.nlm.nih.gov/nucleotide?term=546195%5BBioProject%5D>

### Competing interests

The authors declare that they have no competing interests.

### Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

### Author's contributions

NB and NSR designed and planned the study. JF, KM, NSR, DW and PVDB collected the samples. JF, HM, HMBS, SA, GG, MLM, RHL, PP and NB performed data analyses and interpretation. JF and NB draft the manuscript. All authors read and approved the final manuscript.

### Acknowledgments

Many thanks go to the team of the Institute of Animal Pathology, University of Bern, namely Kerstin Hahn, Corinne Gurtner and Marion Schediwy for performing necropsy and organ sampling of the captive bats in Switzerland. We thank Theresa Pesch, Barbara Prähauser, Lea Rohner, Benita Pineroli and Nadine Jahn for their technical assistance with laboratory work, which was partly performed using the logistics of the Center for Clinical Studies at the Vetsuisse Faculty of the University of Zurich. We also thank Gudrun Wibbelt for the provision of bat samples from Germany and Costa Rica and Caspar Bijleveld und Ursula Kuster for the provision of bat samples from the Papiliorama in Kerzers.

## Abbreviations

bp = base pair

1. = *Chlamydia*

FFPE = formalin-fixed, paraffin-embedded

1. = *Mycoplasma*

rRNA = ribosomal RNA

## References

- (1) Mühldorfer K. Bats and Bacterial Pathogens: A Review. *Zoonoses Public Health*. 2013;60:93–103. doi:10.1111/j.1863-2378.2012.01536.x.
- (2) Kuzmin IV, Bozick B, Guagliardo SA, Kunkel R, Shak JR, Tong S, et al. Bats, emerging infectious diseases, and the rabies paradigm revisited. *Emerg Health Threats J*. 2011;4:7159. doi:10.3402/ehth.v4i0.7159.
- (3) Rurangirwa FR, Dilbeck PM, Crawford TB, McGuire TC, McElwain TF. Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order *Chlamydiales*: proposal of *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. *Int J Syst Bacteriol*. 1999;49 Pt 2:577–581. doi:10.1099/00207713-49-2-577.
- (4) Everett KD, Bush RM, Andersen AA. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol*. 1999;49 Pt 2:415–440. doi:10.1099/00207713-49-2-415.

- (5) Taylor-Brown A, Vaughan L, Greub G, Timms P, Polkinghorne A. Twenty years of research into *Chlamydia*-like organisms: a revolution in our understanding of the biology and pathogenicity of members of the phylum *Chlamydiae*. *Pathog Dis*. 2015;73:1–15. doi:10.1093/femspd/ftu009.
- (6) Greub G, Raoult D. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*. An electron micrograph study. *Appl Environ Microbiol*. 2002;68:3076–3084. doi:10.1128/aem.68.6.3076-3084.2002.
- (7) Chua PKB, Corkill JE, Hooi PS, Cheng SC, Winstanley C, Hart CA. Isolation of *Waddlia malaysiensis*, A Novel Intracellular Bacterium, from Fruit Bat (*Eonycteris spelaea*). *Emerg Infect Dis*. 2005;11:271–277. doi:10.3201/eid1102.040746.
- (8) Pierlé SA, Morales CO, Martínez LP, Ceballos NA, Rivero JJP, Díaz OL, et al. Novel *Waddlia* Intracellular Bacterium in *Artibeus intermedius* Fruit Bats, Mexico. *Emerg Infect Dis*. 2015;21:2161–2163. doi:10.3201/eid2112.150002.
- (9) Hokynar K, Vesterinen EJ, Lilley TM, Pulliainen AT, Korhonen SJ, Paaavonen J, et al. Molecular Evidence of *Chlamydia*-Like Organisms in the Feces of *Myotis daubentonii* Bats. *Appl Environ Microbiol*. 2017;83(2). pii: e02951-16. doi:10.1128/AEM.02951-16.
- (10) Messick JB. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol*. 2004;33:2–13.
- (11) Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed.). *The Prokaryotes. Firmicutes and Tenericutes*. 4. ed. Springer, Berlin, Heidelberg;2014.
- (12) Museux K, Boretti FS, Willi B, Riond B, Hoelzle K, Hoelzle LE, et al. *In vivo* transmission studies of '*Candidatus Mycoplasma turicensis*' in the domestic cat. *Vet Res*. 2009;40:45. doi:10.1051/vetres/2009028.
- (13) Dean RS, Helps CR, Gruffydd Jones TJ, Tasker S. Use of real-time PCR to detect *Mycoplasma haemofelis* and '*Candidatus Mycoplasma haemominutum*' in the saliva and salivary glands of haemoplasma-infected cats. *J Feline Med Surg*. 2008;10:413–417. doi:10.1016/j.jfms.2007.12.007.
- (14) Willi B, Boretti FS, Cattori V, Tasker S, Meli ML, Reusch C, et al. Identification, Molecular Characterization, and Experimental Transmission of a New Hemoplasma Isolate from a Cat with Hemolytic Anemia in Switzerland. *J Clin Microbiol*. 2005;43:2581–2585. doi:10.1128/JCM.43.6.2581-2585.2005.
- (15) Hoelzle LE. Haemotropic mycoplasmas: Recent advances in *Mycoplasma suis*. *Vet Microbiol*. 2008;130:215–226. doi:10.1016/j.vetmic.2007.12.023.
- (16) Dieckmann SM, Winkler M, Groebel K, Dieckmann MP, Hofmann-Lehmann R, Hoelzle K, et al. Haemotropic *Mycoplasma* infection in horses. *Vet Microbiol*. 2010;145:351–353. doi:10.1016/j.vetmic.2010.04.009.
- (17) Maggi RG, Chitwood MC, Kennedy-Stoskopf S, DePerno CS. Novel hemotropic *Mycoplasma* species in white-tailed deer (*Odocoileus virginianus*). *Comp Immunol Microbiol Infect Dis*. 2013;36:607–611. doi:10.1016/j.cimid.2013.08.001.
- (18) Willi B, Filoni C, Catão-Dias JL, Cattori V, Meli ML, Vargas A, et al. Worldwide Occurrence of Feline Hemoplasma Infections in Wild Felid Species. *J Clin Microbiol*. 2007;45:1159–1166. doi:10.1128/JCM.02005-06.
- (19) Yang D, Tai X, Qiu Y, Yun S. Prevalence of *Eperythrozoon* spp. infection and congenital eperythrozoonosis in humans in Inner Mongolia, China. *Epidemiol Infect*. 2000;125:421–426.
- (20) Ikeda P, Seki MC, Carrasco AOT, Rudiak LV, Miranda JMD, Gonçalves SMM, et al. Evidence and molecular characterization of *Bartonella* spp. and hemoplasmas in neotropical bats in Brazil. *Epidemiol Infect*. 2017;145:2038–2052. doi:10.1017/S0950268817000966.
- (21) Mascarelli PE, Keel MK, Yabsley M, Last LA, Breitschwerdt EB, Maggi RG. Hemotropic mycoplasmas in little brown bats (*Myotis lucifugus*). *Parasit Vectors*. 2014;7:117. doi:10.1186/1756-3305-7-117.
- (22) Millán J, López-Roig M, Delicado V, Serra-Cobo J, Esperón F. Widespread infection with hemotropic mycoplasmas in bats in Spain, including a hemoplasma closely related to "*Candidatus Mycoplasma hemohominis*". *Comp Immunol Microbiol Infect Dis*. 2015;39:9–12.

doi:10.1016/j.cimid.2015.01.002.

- (23) Volokhov DV, Becker DJ, Bergner LM, Camus MS, Orton RJ, Chizhikov VE, et al. Novel hemotropic mycoplasmas are widespread and genetically diverse in vampire bats. *Epidemiol Infect.* 2017;145:3154–3167. doi:10.1017/S095026881700231X.
- (24) Pillonel T, Bertelli C, Salamin N, Greub G. Taxogenomics of the order *Chlamydiales*. *Int J Syst Evol Microbiol.* 2015;65:1381–1393. doi:10.1099/ijs.0.000090.
- (25) Millán J, Cevitanes A, Sacristán I, Alvarado-Rybak M, Sepúlveda G, Ramos-Mella CA, et al. Detection and Characterization of Hemotropic Mycoplasmas in Bats in Chile. *J Wildl Dis.* 2019;55:977–981.
- (26) Dietrich M, Mühldorfer K, Tortosa P, Markotter W. Leptospira and Bats. Story of an Emerging Friendship. *PLoS Pathog.* 2015;11:e1005176. doi:10.1371/journal.ppat.1005176.
- (27) Stuckey MJ, Chomel BB, Fleurieu EC de, Aguilar-Setién A, Boulouis H-J, Chang C-C. Bartonella, bats and bugs. A review. *Comp Immunol Microbiol Infect Dis.* 2017;55:20–29. doi:10.1016/j.cimid.2017.09.001.
- (28) Hornok S, Szőke K, Estók P, Krawczyk A, Haarsma A-J, Kováts D, et al. Assessing bat droppings and predatory bird pellets for vector-borne bacteria. Molecular evidence of bat-associated *Neorickettsia* sp. in Europe. *Antonie Van Leeuwenhoek.* 2018;111:1707–1717. doi:10.1007/s10482-018-1043-7.
- (29) Wheelhouse N, Sait M, Gidlow J, Deuchande R, Borel N, Baily J, et al. Molecular detection of *Chlamydia*-like organisms in cattle drinking water. *Vet Microbiol.* 2011;152:196–199. doi:10.1016/j.vetmic.2011.03.040.
- (30) Corsaro D, Müller K, Wingender J, Michel R. "*Candidatus* Mesochlamydia elodeae" (*Chlamydiae: Parachlamydiaceae*), a novel chlamydia parasite of free-living amoebae. *Parasitol Res.* 2013;112:829–838. doi:10.1007/s00436-012-3213-2.
- (31) Codony F, Fittipaldi M, López E, Morató J, Agustí G. Well Water as a Possible Source of *Waddlia chondrophila* Infections. *Microbes Environ.* 2012;27:529–532.
- (32) Hokynar K, Sormunen JJ, Vesterinen EJ, Partio EK, Lilley T, Timonen V, et al. *Chlamydia*-Like Organisms (CLOs) in Finnish *Ixodes ricinus* Ticks and Human Skin. *Microorganisms.* 2016;4. doi:10.3390/microorganisms4030028.
- (33) Pilloux L, Aeby S, Gaumann R, Burri C, Beuret C, Greub G. The High Prevalence and Diversity of *Chlamydiales* DNA within *Ixodes ricinus* Ticks Suggest a Role for Ticks as Reservoirs and Vectors of *Chlamydia*-Related Bacteria. *Appl Environ Microbiol.* 2015;81:8177–8182. doi:10.1128/AEM.02183-15.
- (34) Croxatto A, Rieille N, Kernif T, Bitam I, Aeby S, Péter O, et al. Presence of *Chlamydiales* DNA in ticks and fleas suggests that ticks are carriers of *Chlamydiae*. *Ticks Tick Borne Dis.* 2014;5:359–365. doi:10.1016/j.ttbdis.2013.11.009.
- (35) Thiévent K, Szentiványi T, Aeby S, Glaizot O, Christe P, Greub G. 2019. Presence of *Chlamydia*-like organisms (CLOs) in *Spinturnix myoti*, an ectoparasite of bats. Abstr P-105 Annual Congress of the Swiss Society for Microbiology, Zurich, Switzerland.
- (36) Everett KD, Hornung LJ, Andersen AA. Rapid detection of the *Chlamydiaceae* and other families in the order *Chlamydiales*: three PCR tests. *J Clin Microbiol.* 1999;37:575–580.
- (37) Ehrlich R, Slickers P, Goellner S, Hotzel H, Sachse K. Optimized DNA microarray assay allows detection and genotyping of single PCR-amplifiable target copies. *Mol Cell Probes.* 2006;20:60–63. doi:10.1016/j.mcp.2005.09.003.
- (38) Blumer S, Greub G, Waldvogel A, Hässig M, Thoma R, Tschuor A, et al. *Waddlia*, *Parachlamydia* and *Chlamydiaceae* in bovine abortion. *Vet Microbiol.* 2011;152:385–393. doi:10.1016/j.vetmic.2011.05.024.
- (39) Pospischil A, Kaiser C, Hofmann-Lehmann R, Lutz H, Hilbe M, Vaughan L, et al. Evidence for *Chlamydia* in wild mammals of the Serengeti. *J Wildl Dis.* 2012;48:1074–1078. doi:10.7589/2011-10-298.
- (40) Blumer C, Zimmermann DR, Weilenmann R, Vaughan L, Pospischil A. *Chlamydiae* in free-ranging and captive frogs in Switzerland. *Vet Pathol.* 2007;44:144–150. doi:10.1354/vp.44-2-144.

- (41) Lienard J, Croxatto A, Aeby S, Jatou K, Posfay-Barbe K, Gervais A, et al. Development of a New *Chlamydiales*-Specific Real-Time PCR and Its Application to Respiratory Clinical Samples. *J Clin Microbiol.* 2011;49:2637–2642. doi:10.1128/JCM.00114-11.
- (42) Willi B, Meli ML, Lüthy R, Honegger H, Wengi N, Hoelzle LE, et al. Development and application of a universal Hemoplasma screening assay based on the SYBR green PCR principle. *J Clin Microbiol.* 2009;47:4049–4054. doi:10.1128/JCM.01478-09.
- (43) Tasker S, Peters IR, Mumford AD, Day MJ, Gruffydd-Jones TJ, Day S, et al. Investigation of human haemotropic Mycoplasma infections using a novel generic haemoplasma qPCR assay on blood samples and blood smears. *J Med Microbiol.* 2010;59:1285–1292. doi:10.1099/jmm.0.021691-0.
- (44) Gouy M, Guindon S, Gascuel O. SeaView version 4. A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol.* 2010;27:221–224. doi:10.1093/molbev/msp259.
- (45) Thiévent K, Szentiványi T, Aeby S, Glaizot O, Christe P, Greub G. 2019. Presence of *Chlamydia*-like organisms (CLOs) in *Spinturnix myoti*, an ectoparasite of bats. Abstr P-105 Annual Congress of the Swiss Society for Microbiology, Zurich, Switzerland.

## Tables

Table 1: Sampled bats categorized according to origin, family, species and number of animals.

Origin		Family	Species	Number of Animals		
Captive	Switzerland	<i>Phyllostomidae</i>	<i>Carollia perspicillata</i>	89		
	Germany	<i>Phyllostomidae</i>	<i>Carollia perspicillata</i>	13		
			<i>Glossophaga commissarisi</i>	2		
			<i>Glossophaga soricina</i>	2		
			<i>Phyllostomus discolor</i>	5		
			<i>Pteropodidae</i>	<i>Eidolon helvum helvum</i>	4	
			<i>Rousettus aegyptiacus</i>	1		
		<i>Megadermatidae</i>	<i>Megaderma lyra</i>	1		
	Free-living	Switzerland	<i>Vespertilionidae</i>	<i>Eptesicus nilssonii</i>	1	
				<i>Eptesicus sp.</i>	1	
<i>Hypsugo savii</i>				1		
<i>Myotis daubentonii</i>				2		
<i>Myotis myotis</i>				18		
<i>Myotis mystacinus</i>				8		
<i>Myotis nattereri</i>				2		
<i>Nyctalus leisleri</i>				2		
<i>Nyctalus noctula</i>				6		
<i>Pipistrellus kuhlii</i>				28		
<i>Pipistrellus nathusii</i>				18		
<i>Pipistrellus pipistrellus</i>				123		
<i>Pipistrellus pygmaeus</i>				4		
<i>Pipistrellus sp.</i>				47		
<i>Plecotus auritus</i>				4		
<i>Plecotus sp.</i>				3		
<i>Vespertilio murinus</i>				8		
				<i>Rhinolophidae</i>	<i>Rhinolophus ferrumequinum</i>	2
					<i>Rhinolophus hipposideros</i>	2
				<i>Unknown</i>	<i>Unknown</i>	5
Germany	<i>Vespertilionidae</i>	<i>Barbastella barbastellus</i>	2			
		<i>Eptesicus nilssonii</i>	6			
		<i>Eptesicus serotinus</i>	15			
		<i>Nyctalus leisleri</i>	1			
		<i>Nyctalus noctula</i>	16			
		<i>Plecotus auritus</i>	7			
		<i>Vespertilio murinus</i>	9			
Costa Rica	<i>Phyllostomidae</i>	<i>Artibeus watsoni</i>	1			

	<i>Carollia perspicillata</i>	1
	<i>Glossophaga commissarisi</i>	13
<i>Vespertilionidae</i>	<i>Rhogeessa io</i>	1
<i>Emballonuridae</i>	<i>Saccopteryx bilineata</i>	1
<b>Total</b>		<b>475</b>

Table 2: Sequencing results of *Chlamydiales* and hemotropic mycoplasmas positive bats including best BLAST and identity results.

*Chlamydiales*

Sample ID	bat species & origin	Method	best BLAST	identity
52 (spleen)	<i>Carollia perspicillata</i> (Switzerland, captive)	16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone SU16A24ocu 16S ribosomal RNA gene, partial sequence (MH119787.1)	99.12%
95 (liver)	<i>Carollia perspicillata</i> (Switzerland, captive)	16S-pan-PCR	Uncultured <i>Chlamydiae</i> bacterium clone Upland_500_9569 16S ribosomal RNA gene, partial sequence (JF990268.1)	95.00%
F18-0155.54 (FFPE)	<i>Myotis myotis</i> (Switzerland, free-living)	16S-IGF/IGR-PCR	<i>Chlamydia pecorum</i> gene for 16S rRNA, strain B0-Maeda (AB001775.1)	93.29%
		16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1778 16S ribosomal RNA gene, partial sequence (KU664271.1)	98.98%
F18-0155.98 (FFPE)	<i>Pipistrellus pipistrellus</i> (Switzerland, free-living)	16S-IGF/IGR-PCR	<i>Chlamydia trachomatis</i> strain SQ24 chromosome, complete genome (CP017733.1)	93.16%
		16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1778 16S ribosomal RNA gene, partial sequence (KU664271.1)	98.98%
F18-0155.121 (FFPE)	<i>Pipistrellus</i> sp. (Switzerland, free-living)	16S-IGF/IGR-PCR	Uncultured <i>Chlamydiales</i> bacterium clone KP5_Horse 16S ribosomal RNA gene, partial sequence (MK112598.1)	92.36%
		16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1778 16S ribosomal RNA gene, partial sequence (KU664271.1)	99.49%
F18-0155.130 (FFPE)	<i>Pipistrellus pipistrellus</i> (Switzerland, free-living)	16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone cafs1013 16S ribosomal RNA gene, partial sequence (MF440154.1)	100.00%
F18-0155.145 (FFPE)	<i>Pipistrellus pipistrellus</i> (Switzerland, free-living)	16S-pan-PCR	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 11Aug11-23 (LC336010.1)	100.00%
E 4/08 (intestine)	<i>Glossophaga soricina</i> (Germany, captive)	16S-pan-PCR	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 07Nov4-93 (AB618438.1)	97.42%
E 6/08 (intestine)	<i>Glossophaga soricina</i>	16S-pan-PCR	Uncultured bacterium clone BS.DW1.262 16S ribosomal RNA gene, partial sequence (KY692835.1)	96.86%

	(Germany, captive)			
E 148/07 (intestine)	<i>Nyctalus noctula</i> (Germany, free-living)	16S-IGF/IGR-PCR	<i>Chlamydia pecorum</i> gene for 16S rRNA, strain B0-Maeda (AB001775.1)	93.51%
		23SIG-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1703 23S ribosomal RNA gene, partial sequence (KU664232.1)	95.78%
E 155/07 (intestine)	<i>Nyctalus noctula</i> (Germany, free-living)	16S-pan-PCR	<i>Chlamydia</i> sp. S15-834C isolate S15-834K genome assembly, chromosome: I (LS992154.1)	95.79%
E 161/07 (intestine)	<i>Eptesicus serotinus</i> (Germany, free-living)	16S-IGF/IGR-PCR	<i>Chlamydia pecorum</i> gene for 16S rRNA, strain B0-Maeda (AB001775.1)	94.09%
		23SIG-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1703 23S ribosomal RNA gene, partial sequence (KU664232.1)	95.79%
		16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone 1778 16S ribosomal RNA gene, partial sequence (KU664271.1)	98.98%
E 179/07 (intestine)	<i>Nyctalus noctula</i> (Germany, free-living)	16S-pan-PCR	<i>Chlamydiales</i> bacterium isolate CL gt1 16S ribosomal RNA gene, partial sequence (MF620054.1)	97.51%
E 197/07 (intestine)	<i>Vespertilio murinus</i> (Germany, free-living)	16S-pan-PCR	Uncultured <i>Chlamydiales</i> bacterium clone HE210050 16S ribosomal RNA gene, partial sequence (HQ721227.1)	99.37%
E 18/07 (intestine)	<i>Rhogeessa io</i> (Costa Rica, free-living)	16S-pan-PCR	<i>Rhabdochlamydiaceae</i> bacterium isolate P gt1 16S ribosomal RNA gene, partial sequence (MF620051.1)	96.60%

**Hemotropic Mycoplasmas**

Sample ID	bat species & origin	Method	best BLAST	identity
E 173/07 (spleen)	<i>Nyctalus noctula</i> (Germany, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured <i>Mycoplasma</i> sp. clone 20180131LOC1.16 16S ribosomal RNA gene, partial sequence (MK295631.1)	97.41%
E 190/07 (spleen)	<i>Vespertilio murinus</i> (Germany, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured <i>Mycoplasma</i> sp. clone 20180131LOC1.16 16S ribosomal RNA gene, partial sequence (MK295631.1)	98.60%
E 70/06 (spleen)	<i>Glossophaga commissarisi</i> (Costa Rica, free-living)	HemMycop41/938-PCR	Uncultured <i>Mycoplasma</i> sp. clone D159 16S ribosomal RNA gene, partial sequence (KY932722.1)	96.25%

Others

F18-0155.55 (FFPE)	<i>Pipistrellus kuhlii</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured bacterium clone B17Sof4_14toy 16S ribosomal RNA gene, partial sequence (MK372594.1)	99.44%
F18-0155.63 (FFPE)	<i>Pipistrellus pipistrellus</i> (Switzerland, free-living)	HemMycop41/938-PCR	Uncultured bacterium clone B17Sof4_14toy 16S ribosomal RNA gene, partial sequence (MK372594.1)	99.46%
F18-0155.70 (FFPE)	<i>Nyctalus noctula</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured <i>Sphingomonadaceae</i> bacterium clone Elev_16S_810 16S ribosomal RNA gene, partial sequence (EF019656.1)	92.81%
F18-0155.99 (FFPE)	<i>Pipistrellus kuhlii</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured bacterium clone B17Sof4_14toy 16S ribosomal RNA gene, partial sequence (MK372594.1)	99.63%
F18-0155.102 (FFPE)	<i>Pipistrellus kuhlii</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	<i>Bradyrhizobium</i> sp. strain B2_2 16S ribosomal RNA gene, partial sequence (MH086239.1)	98.81%
F18-0155.110 (FFPE)	<i>Myotis daubentonii</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	<i>Altererythrobacter</i> sp. strain S1-5 16S ribosomal RNA gene, partial sequence (MK574878.1)	92.72%
F18-0155.113 (FFPE)	<i>Nyctalus noctula</i> (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	<i>Bradyrhizobium</i> sp. strain aclo_27 16S ribosomal RNA gene, partial sequence (MG588424.1)	89.52%
F18-0155.116 (FFPE)	<i>Pipistrellus</i> sp. (Switzerland, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	<i>Bradyrhizobium</i> sp. strain BRUESC966 16S ribosomal RNA gene, partial sequence (MH636098.1)	99.89%
E 196/07 (spleen)	<i>Eptesicus nilssonii</i> (Germany, free-living)	HemMycop41/938-PCR HemMycop322/1420-PCR	Uncultured bacterium clone B17Sof4_14toy 16S ribosomal RNA gene, partial sequence (MK372594.1)	98.91%

Table 3: Primers and probes used in the present study.

PCR method*	Gene target & amplicon size	Name	Sequence (5' - 3')	Reference	
Chlam23S-qPCR	23S rRNA, 111 bp	Ch23S-F	CTGAAACCAGTAGCTTATAAGCGGT	Ehrlich et al. 2006	
		Ch23S-R	ACCTCGCCGTTTAACTTAACTCC		
		Ch23S-p	6-FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA		
23SIG-PCR	23S rRNA signature sequence, 700 bp	U23-F	GATGCCTTGGCATTGATAGGCGATGAAGGA	Everett et al. 1999	
		23SIG-R	TGGCTCATCATGCAAAAGGCA		
16S-IGF/IGR-PCR	16S rRNA, 278 bp	16S-IGF	GATGAGGCATGCAAGTCGAACG	Pospischil et al. 2012; Blumer et al. 2007	
		16S-IGR	CCAGTGTTGGCGGTCAATCTCTC		
16S-pan-qPCR <sup>1</sup>	16S rRNA, 200 bp <sup>1,2</sup>	16S-panCh-F <sup>1,2</sup>	CCGCAACACTGGGACT	Lienard et al. 2011 <sup>1</sup> , Hokynar et al. 2016 <sup>2</sup>	
16S-pan-PCR <sup>2</sup>		16S-panCh-R <sup>1,2</sup>	GGAGTTAGCCGGTGCTTCTTTAC		
		16S-panCh <sup>1</sup>	6-FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1		
		sequencing primers <sup>1</sup>	panFseq <sup>1</sup>		CCAACACTGGGACTGAGA
			panRseq <sup>1</sup>		GCCGGTGCTTCTTTAC
eGFP-qPCR	eGFP, 177 bp	eGFP-1-F	GACCACTACCAGCAGAACAC		
		eGFP-10-R	CTTGTACAGCTCGTCCATGC		
		eGFP-Hex	VIC-AGCACCCAGTCCGCCCTGAGCA-none		
Hemoplasma SYBR Green qPCR	16S rRNA	Mhae_sybr.359f	AGCAATACCATGTGAACGATGAA	Willi et al. 2009	
		Mcocc_sybrF	AGCAATGCCATGTGAACGATGAA		
		Mhae_sybr.432r	TGGCACATAGTTTGCTGTCACTT		
		Cmhae_Sybr.493r	GCTGGCACATAGTTAGCTGTCACT		
Mhf-like qPCR	16S rRNA, 114 bp	Group_Mhf_fwd	GGAGCGGTGGAATGTGTAG	Tasker et al. 2010	
		Group_Mhf_rev	GGGGTATCTAATCCCATTTGC		
		Group_Mhf_probe	6-FAM-TYAAGAACACCAGAGGCCGAAGGCCG-BHQ1		
CMmh-like qPCR	16S rRNA, 139 bp	Group_CMhm_fwd	GGGGCCAAGTCAAGTCATC	Tasker et al. 2010	
		Group_CMhm_rev	GCGAATTGCAGCCTTTTATC		
		Group_CMhm_probe	YYE-TACCATTGTAGCACGTTYGCAGCCC-		

			BHQ1	
HemMycop41/938-PCR	16S rRNA, 871 bp	HemMycop16S-41s	GYATGCMTAAYACATGCAAGTCGARCG	Mascarelli et al. 2014
		HemMycop16S-938as	CTCCACCACTTGTTCAGGTCCCCGTC	
HemMycop322/1420-PCR	16S rRNA, 1030 bp	HemMycop16S-322s	GCCCATATTCTACGGGAAGCAGCAGT	Mascarelli et al. 2014
		HemMycop16S-1420as	GTTTGACGGGCGGTGTGTACAAGACC	

\*conventional PCR (PCR), real-time PCR (qPCR)

1 & 2 PCRs performed according to the respective labeled reference, using the respective labeled primers

## Figures

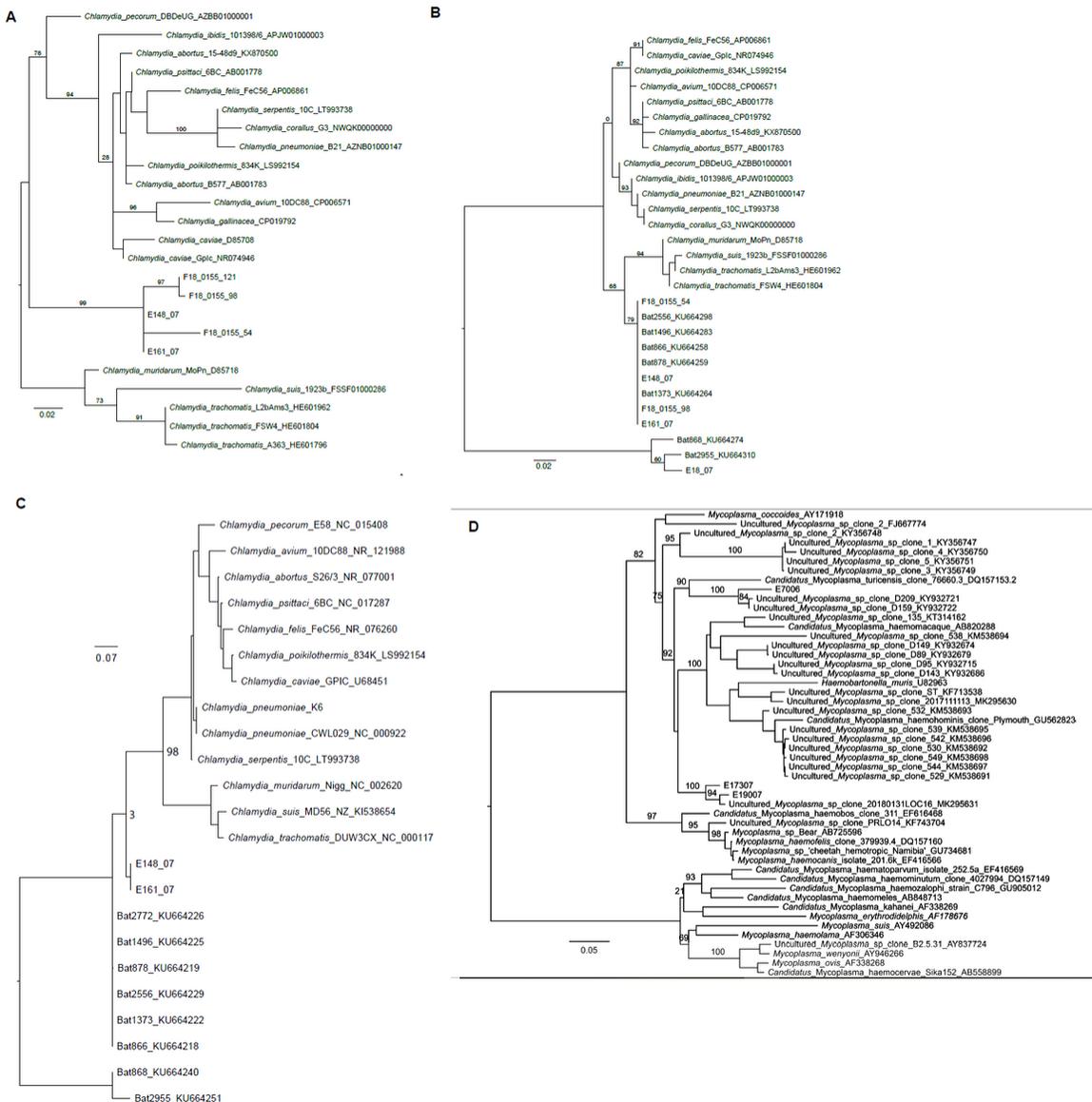


Figure 1

Phylogenetic trees showing the relationships of the sequences obtained in this study and publicly available sequences of Chlamydia or Mycoplasma species. A. Phylogeny of chlamydial 16S rRNA gene, 284 bp covering V1 – V2, including all novel sequences and illustrating that they fall together within a novel clade. B. Phylogeny of chlamydial 16S rRNA gene, 200 bp covering V3, relating available novel sequences to those previously found in bat samples and illustrating that these samples are closely related to previous bat samples over this region. C. Phylogeny of chlamydial 23S rRNA gene, 530 bp, relating available novel sequences to those previously found in bat samples and illustrating differences between the novel samples and previous bat samples across this region. Bat samples were selected from those published in Hokynar et al. (9) to reflect closely related samples and outgroup "Rhabdochlamydiaceae-like" samples. D. Phylogenetic tree based on concatenated sequences obtained from PCR products of the gene encoding for the 16S rRNA gene of hemotropic mycoplasmas, 1252 bp (minimum 824 bp). Bootstraps of 100 replicates are shown on key branches. Scale bar shows number of substitutions per site.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1final.pdf](#)