Prevalence of hemoplasmas and Bartonella species in client-owned cats in Beijing and Shanghai, China

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Running head: FELINE HEMOPLASMAS AND BARTONELLA IN CHINA
ABSTRACT

A year-round molecular epidemiological survey (2017 to 2018) was conducted on three hemoplasmas and two Bartonella species with zoonotic potential in client-owned cats in Beijing and Shanghai. Among 668 specimens, the overall hemoplasma-positive rate was 4.9% (3.4% for Candidatus Mycoplasma haemominutum, 0.9% for Mycoplasma haemofelis and 1.2% for Candidatus Mycoplasma turicensis). The overall Bartonella-positive rate was 8.5% (4.8% for B. henselae and 4.3% for B. clarridgeiae). Age, breed, ectoparasiticide use and stray history, but not city, season and gender, were significantly associated with the positive rates of one or more pathogens. This is also the first report on the prevalence of Candidatus Mycoplasma turicensis in cats in China.

Keywords: Bartonella, China, feline, hemoplasma, risk factor
 Hemoplasmas (aka hemotropic mycoplasmas) and *Bartonella* are vector-transmitted gram-negative bacterial pathogens in animals. Hemoplasmas adhere to and disrupt erythrocytes, causing hemolytic anemia in animals. Cats may be infected by *Mycoplasma haemofelis* (*Mhf*), “*Candidatus Mycoplasma haemominutum*” (*CMhm*), “*Candidatus Mycoplasma turicensis*” (*CMt*), and “*Candidatus Mycoplasma haematoparvum*-like” (*CMhp*) species [18, 19]. Among them, *Mhf* may cause severe to fatal hemolytic anemia in cats. *CMhm* is typically low virulent, but can cause severe clinical signs when co-infected with other pathogens and/or if the animal is under stressed or immunodeficient condition [18]. *CMt* can induce mild to moderate anemia in experimentally infected cats in the acute infection phase [24], while the clinical significance of *CMhp* is not fully understood.

*Bartonella* species are intracellular pathogens infecting animals including cats and dogs. Cats can serve as reservoir host for *B. henselae, B. clarridgeiae* and *B. koehlerae*. Among them, *B. henselae* and *B. clarridgeiae* are the causative agents of cat scratch disease (CSD) in humans [15]. However, naturally infected cats usually exhibit no clinical signs even after long-term experience of bacteremia [10]. Fleas are believed to be the predominant vector responsible for the transmission of *Bartonella* species.

Despite their importance in animal health and zoonotic potential, there were limited studies on the prevalence of feline hemoplasma and *Bartonella* in China. The presence of feline *Mhf* and *CMhm* in the mainland China was first reported in 2010, in which the scale of the study was limited and the prevalence of *CMt* was not evaluated [27]. A few other studies investigated the prevalence of *Bartonella* in stray or pet cats in some regions in China [25, 26]. However, the prevalence of feline hemoplasma and *Bartonella* in Beijing, the nation’s capital with high population densities of human residents and pets, has not been reported.

In the present study, we conducted a year-round molecular survey between 2017 and 2018 on the prevalence of three hemoplasmas (*Mhf, CMhm* and *CMt*) and two *Bartonella* species (*B. henselae* and *B. clarridgeiae*) in client-owned cats in Beijing and Shanghai, two of the most populated cities in the north and south regions in China, and analyzed associated risk factors to expand epidemiological information.

For specimen collection, a total of 668 blood samples were collected from client-owned cats at four veterinary hospitals in Beijing and one in Shanghai between March, 2017 to March, 2018. Specimens were shipped to the College of Veterinary Medicine, China Agricultural
University for storage at -20 °C until use. During sample collection, the following information on cats was recorded by veterinarians or collected from clients: city (Beijing, Shanghai), season (spring, summer, autumn, winter), age (≤1 year, 1-10 years, ≥10 years), gender (male, female), breed (purebreds, mixed including crossbreeds or unknown breeds), stray history, and ectoparasiticide use in the past 6 months. The animal use protocol was reviewed and approved by the Laboratory Animal Welfare and Animal Experimental Ethics Committee, China Agricultural University (permit number: AW21012020-2). Prior to specimen collection, permission was obtained from animal owners.

For molecular detection by PCR, genomic DNA was extracted from 200 μl of each blood sample using a QIAamp DNA Blood Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany), eluted in 100 μl elution buffer and stored at -20 °C until use. Hemoplasmas and Bartonella species were detected by nested PCR that amplified a 16S-23S rRNA intergenic transcribed spacer (ITS). Primary PCR used genus-specific primers, while secondary PCR used species-specific primers as described (Supplementary Table 1) [12, 16, 22-23]. PCR was performed in 25 μl volume containing 12.5 μl of 2× PCR Starmix (GenStar BioSolutions, Beijing), 1.0 μM each of specified primers and 2.0 μl sample DNA for primary PCR (or 1.0 μl primary PCR product for secondary PCR), using thermal cycling conditions described in Supplementary Table 1. DNA elution buffer and hemoplasma or Bartonella DNA samples were used for negative and positive controls, respectively. PCR products were electrophoresed in 2% agarose gels. All samples were tested at least twice. PCR products were extracted from gels and submitted to Beijing Majorbio Sanger Bio-pharm Technology for bi-directional automated sequencing using ABI Prism 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Hemoplasmas and Bartonella DNAs detected in the present study were compared with genomic sequences in the GenBank by BLAST search.

The association between individual risk factors and infections of hemoplasma or Bartonella was evaluated by Chi-square (χ²) test or by a two-tailed Fisher’s exact test when expected numbers of observations were less than five using SPSS (version 20). Variables with P-values < 0.05 in the univariant analysis were further tested using a multivariable logistic regression model. A P-value <0.05 was considered statistically significant in both univariate and multivariate analyses.

The results showed that the overall positive detection rate by nested PCR for hemoplasmas was 4.9% (n = 33 of the total 668 specimens) (Table 1). Among them, CMhm was the most
prevalent species (n = 23; 3.4%), followed by CMt (n = 8; 1.2%) and Mhf (n = 6; 0.9%).

Coinfections by two hemoplasma species were observed in some cats, including three specimens with CMhm and Mhf (0.4%) and one with Mhf and CMt (0.1%), but none with three hemoplasma species (i.e., CMhm, Mhf and CMt). The positive rate of hemoplasmas (i.e., 4.9%) is much lower than an earlier study conducted in a southern city Guangzhou, China, (i.e., 41.4%) [27], and those reported in other countries, including Japan (26.4%) [21], Thailand (38.1%) [3], South Korea (47.9%) [6], Iran (22%) [4], the United States (18% to 27%) [19, 20], and the United Kingdom (18% to 27%) [14].

The overall positive detection rate for Bartonella spp. in this study was 8.5% (n = 57 of the total 668 specimens), which was much higher than that of hemoplasmas. It included 4.8% (n = 32) for B. henselae and 4.3% (n = 29) for B. clarridgeiae. Among them, 0.6% (n = 4) of the Bartonella-positive specimens were coinfect ed both species (Table 1). The overall positive rate of 8.5% was between the two values reported earlier for feline Bartonella in other regions in China, i.e., 3.9% in cats from the southern city Shenzhen [26], and 12.7% in cats from 7 provinces (Beijing not included) [25]. In comparison with studies in other countries, the overall prevalence of Bartonella in our study is comparable to those reported in Japan (4.6%) [16], Turkey (9.4%) [1], Greece (8.5%) [13], and Ireland (5.2%) [8], but lower than that those in Thailand (16.3%) [7], South Korea (41.8% to 44.1%) [9] Taiwan (19.1%) [2], and Israel (18.7 to 30.7%) [5].

Our results indicated that hemoplasmas and Bartonella species were commonly present as a potential health risk to cats in China. The presence of zoonotic B. henselae and B. clarridgeiae was also an indication of potential risk for CSD in humans. Indeed, several CSD cases have already been reported in China [11], suggesting the necessity for veterinarians to educate pet owners regarding the risk of CSD in contacting with cats.

In risk factor analysis, we observed no significant differences between Beijing and Shanghai in the overall positive detection rates of feline hemoplasmas or Bartonella (P = 0.285 to 0.856 by the univariant test) (Table 2). Although both bacterial groups had the highest positive rates in the spring, season was not a significant risk factor (P = 0.344 to 0.935). Gender was also not a significant risk factor for infections of hemoplasma or Bartonella species (P = 0.690 to 0.910). However, age, breed, ectoparasiticide use and stray history were significantly associated with the positive detection rates of one or more pathogens (Table 2). For age, significantly higher hemoplasma-positive rates were observed in 1 to 10-year old cats.
cats (8.0%) than in younger (≤1 year) or older (≥10 years) animals (2.6%, \( P = 0.006 \)); while
significantly higher positive rates of \textit{Bartonella} species were observed in both ≤1 year and 1
to 10-year old groups (5.6% to 7.2%) than in older animals (≥10 years) (1.3% to 1.7%, \( P =
0.019 \) to 0.025).

Between the two breed types, mixed breeds had significantly higher positive rates (6.7% to
7.6%) than purebreds (2.1% to 2.6%) for both hemoplasmas and \textit{Bartonella} spp. (\( P = 0.002 \)
to 0.008). Cats with stray history had >2 times higher positive rates (8.0% to 9.1%) than
those without stray history (2.7% to 3.5%, \( P = 0.001 \) to 0.015). Further analysis by multiple
logistic regression indicated that age (\( P = 0.018 \)) and breed (\( P = 0.025 \)) were significantly
associated with hemoplasma infections after the adjustment for stray history (Table 3). Age
(\( P = 0.014 \) and 0.044), stray history (\( P = 0.028 \)) and ectoparasiticide use (\( P = 0.022 \)) were
significantly associated with \textit{B. henselae} infection, while only age (\( P = 0.012 \) and 0.032) was
significantly associated with \textit{B. clarridgeiae} infection (Table 3).

The fact that both hemoplasma and \textit{Bartonella} infections are more frequently observed in
mixed breed cats than purebreds may relate to their living environments, since mixed breed
cats are more likely to be allowed for outdoor activities in China. Outdoor cats are more
prone to the exposure to arthropod vectors that potentially carry pathogens. Another
possibility is that most mixed breed cats are adopted from places where they were housed in
groups with higher chances to socialize with infected-cats. The behavior changes in cats over
the age might also be a contributor to the variation of infection. For instance, higher
hemoplasma-positive rates were observed in 1 to 10-year old cats that are generally more
aggressive in interacting with each other, thus increasing the risk of infection. On the other
hand, older cats (≥10 years) might spend less time roaming outside [17], thus reducing the
risk of vector exposure, resulting in lower infections for both hemoplasmas and \textit{Bartonella}.
Among other risk factors, the significantly higher prevalence of hemoplasmas and \textit{Bartonella}
in cats with stray history, and that of \textit{B. henselae} in cats without ectoparasiticide use, were
apparently related to the higher chances of animal exposure to vectors (e.g., fleas).

We were able to retrieve medical records of 224 sampled cats from veterinarians in Beijing
for analyzing the relationship between infection and anemia, but observed that the overall
hemoplasma-positive rate was not statistically different between cats with and without
anemia (9.2% vs. 12.9%; \( P = 0.372 \)) (Table 2). This observation suggested that hemoplasma
infection contributed no more than other possible factors to the overall rate of feline anemia
in Beijing. However, there is a lack of more direct evidence on the relationship between hemoplasma-infection and anemia because the medical records were not individually paired between anemia status and hemoplasma infection. We could only retrieve four paired records, showing three (75%) of the four Mhf-positive cats had anemia, weakly supporting Mhf as a potential cause of feline anemia.

In summary, our year-round survey between 2017 and 2018 indicated the presence of three hemoplasmas and two Bartonella species in cats in two Chinese metropolitan cities. Age of animals and their stray history are the two major factors positively associated with the infection rates. The presence of zoonotic Bartonella species indicates a significant risk for CSD in humans.

Conflict of interest

The authors declare no conflict of interest with respect to the publication of this manuscript.

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Table 1 PCR results for hemoplasma and *Bartonella* infections in 668 cat samples

| Nested-PCR result (N = 668) | No. of positive cats | Positive rate (%) |
|-----------------------------|----------------------|-------------------|
| **Hemoplasmas**             |                      |                   |
| *CMhm*                      | 23                   | 3.4               |
| *Mhf*                       | 6                    | 0.9               |
| *CMt*                       | 8                    | 1.2               |
| *CMhm* only                 | 20                   | 3.0               |
| *Mhf* only                  | 2                    | 0.3               |
| *CMt* only                  | 7                    | 1.0               |
| *CMhm* + *Mhf*              | 3                    | 0.4               |
| *Mhf* + *CMt*               | 1                    | 0.1               |
| *CMhm* + *Mhf* + *CMt*      | 0                    | 0                 |
| **Bartonella species**      | 57                   | 8.5               |
| *B. henselae*               | 32                   | 4.8               |
| *B. clarridgei*             | 29                   | 4.3               |
| *B. henselae* only          | 28                   | 4.2               |
| *B. clarridgei* only        | 25                   | 3.7               |
| *B. henselae* + *B. clarridgei* | 4      | 0.6               |
Table 2 Univariate analysis of risk factors for infection of feline hemoplasmas and *Bartonella* spp. in cats

| Risk Factors | Total N | Hemoplasmas | B. henselae | B. clarridgeiae |
|-------------|---------|-------------|-------------|----------------|
|             |         | N of positive cats (%) | P  | N of positive cats (%) | P  | N of positive cats (%) | P  |
| City        | 668     | 33 (4.9%) | 0.285 | 32 (4.8%) | 0.580 | 29 (4.3%) | 0.856 |
| Beijing     | 516     | 28 (5.4%) | 0.580 | 26 (5.0%) | 0.267 | 22 (4.3%) | 0.856 |
| Shanghai    | 152     | 5 (3.3%) | 0.856 | 6 (3.9%) | 0.856 | 7 (4.6%) | 0.856 |
| Season      | 668     |           |        |            |        |            |        |
| Spring      | 200     | 13 (6.5%) | 0.360 | 14 (7.0%) | 0.344 | 10 (5.0%) | 0.935 |
| Summer      | 122     | 3 (2.5%) | 0.935 | 5 (4.1%) | 0.935 | 5 (4.1%) | 0.935 |
| Autumn      | 132     | 5 (3.8%) | 0.935 | 4 (3.0%) | 0.935 | 6 (4.5%) | 0.935 |
| Winter      | 214     | 12 (5.6%) | 0.935 | 9 (4.2%) | 0.935 | 8 (3.7%) | 0.935 |
| Age         | 668     |           |        |            |        |            |        |
| ≤1year      | 153     | 4 (2.6%) | 0.006 | 11 (7.2%) | 0.025 | 10 (6.5%) | 0.019 |
| 1-10years   | 286     | 23 (8.0%) | 0.006 | 17 (5.9%) | 0.025 | 16 (5.6%) | 0.019 |
| ≥10years    | 229     | 6 (2.6%) | 0.006 | 4 (1.7%) | 0.025 | 3 (1.3%) | 0.019 |
| Breed       | 668     |           |        |            |        |            |        |
| Mixed breeds| 327     | 25 (7.6%) | 0.002 | 22 (7.0%) | 0.008 | 22 (6.7%) | 0.003 |
| Purebreds   | 341     | 8 (2.3%) | 0.002 | 9 (2.6%) | 0.008 | 7 (2.1%) | 0.003 |
| Gender      | 668     |           |        |            |        |            |        |
| Male        | 403     | 21 (5.2%) | 0.690 | 19 (4.7%) | 0.910 | 17 (4.2%) | 0.848 |
| Female      | 265     | 12 (4.5%) | 0.690 | 13 (4.9%) | 0.910 | 12 (4.5%) | 0.848 |
| Ectoparasitic use | 668 |           |        |            |        |            |        |
| Yes         | 292     | 13 (4.5%) | 0.608 | 8 (2.7%) | 0.029 | 13 (4.5%) | 0.901 |
| No          | 376     | 20 (5.3%) | 0.608 | 24 (6.4%) | 0.029 | 16 (4.3%) | 0.901 |
| Former stray| 668     |           |        |            |        |            |        |
| Yes         | 187     | 17 (9.1%) | 0.002 | 15 (8.0%) | 0.015 | 16 (8.6%) | 0.001 |
| No          | 481     | 16 (3.3%) | 0.002 | 17 (3.5%) | 0.015 | 13 (2.7%) | 0.001 |
| Anemia status| 224 |           |        |            |        |            |        |
| Yes         | 131     | 12 (9.2%) | 0.372 |            |        |            |        |
| No          | 93      | 12 (12.9%) | 0.372 |            |        |            |        |
### Table 3 Multivariate analysis of risk factors for infection of feline hemoplasmas and *Bartonella* spp. in cats

|                          | OR (CI<sub>95</sub>) | P     |
|--------------------------|----------------------|-------|
| **1. Hemoplasmas**       |                      |       |
| Age (n=668)              |                      |       |
| ≤1 year                  | 1.030 (0.283-3.745)  | 0.964 |
| 1-10 years               | 3.099 (1.209-7.943)  | **0.018** |
| ≥10 years                | Ref.                 |       |
| Breed (n=668)            |                      |       |
| Mixed breed              | 2.888 (1.139-7.321)  | **0.025** |
| Purebreds                | Ref.                 |       |
| Former strays (n=668)    |                      |       |
| Yes                      | 1.436 (0.629-3.278)  | 0.390 |
| No                       | Ref.                 |       |
| **2. Bartonella henselae** |                    |       |
| Age (n=668)              |                      |       |
| ≤1 year                  | 4.325 (1.342-13.940) | **0.014** |
| 1-10 years               | 3.147 (1.029-9.627)  | **0.044** |
| ≥10 years                | Ref.                 |       |
| Former strays (n=668)    |                      |       |
| Yes                      | Ref.                 |       |
| No                       | 2.283 (1.093-4.772)  | **0.028** |
| Ectoparasiticide use (n=668) |                  |       |
| Yes                      | 2.610 (1.145-5.949)  | **0.022** |
| No                       | Ref.                 |       |
| **3. Bartonella clarridgeiae** |                   |       |
| Age (n=668)              |                      |       |
| ≤1 year                  | 5.424 (1.452-20.258) | **0.012** |
| 1-10 years               | 3.988 (1.124-14.144) | **0.032** |
| ≥10 years                | Ref.                 |       |
| Breed (n=668)            |                      |       |
| Mixed breed              | 2.631 (0.973-7.116)  | 0.057 |
| Purebreds                | Ref.                 |       |
| Former strays (n=668)    |                      |       |
| Yes                      | Ref.                 |       |
| No                       | 1.878 (0.778-4.532)  | 0.161 |

OR = odds ratio, CI<sub>95</sub> = 95% confidence interval
Supplementary Table 1  Polymerase chain reaction details for the nested PCR assays used in the study for the detection of hemoplasma and *Bartonella* spp.

| RCR | Target        | Primer  | Sequence (5' → 3')                  | Fragment size (bp) | Ref. | Thermal cycling protocols                      |
|-----|---------------|---------|-------------------------------------|--------------------|------|-----------------------------------------------|
|     |               |         |                                     |                    |      |                                               |
|     | Primary       | hemoplasmas | fHf1  | ACGCGTCGA CAGAGTTTG ATCCTGGCT       | 1499 bp            | 12   | 95°C, 5 min; 35 cycles of 45 sec at 95°C, 1 min at 51°C, 2 min at 72°C; 72°C, 7 min |
|     |               |         | rHf2  | CGCGGATCC GCTACCTTG TTACGACTT       |                    |      |                                               |
|     | Secondary     | Mhf     | OH-Ok1 | ATGCCCCCTC TGTTGGGGGA TAGCCG        | 273 bp             | 23   |                                               |
|     |               |         | 00CB-rl | ATGGTATTTG CTCCATCAG ACTTTTCG      |                    |      |                                               |
|     | Secondary     | CMhm    | CA-B2 | CTGGGAAAAA CAATGGTC GGAAG          | 202 bp             | 23   | 95°C, 5 min; 35 cycles of 45 sec at 95°C, 45 sec at 58.4°C, 45 sec at 72°C; 72°C, 7 min |
|     |               |         | 00CB-r1 | ATGGTATTTG CTCCATCAG ACTTTTCG     |                    |      |                                               |
|     | Secondary     | CMt     | CMrR  | TCCTATAGT TCCTCCATC AGACA          | 210 bp             | 22   |                                               |
|     |               |         | 00CB-r1 | ATGGTATTTG CTCCATCAG ACTTTTCG     |                    |      |                                               |
|     | Primary       | Bartonella | URBarto1 | CTTCGTTTCT CTTTCTTCA  | 722 bp             | 16   | 95°C, 5 min; 35 cycles of 45 sec at 95°C, 45 sec at 50°C, 1 min at 72°C; 72°C, 7 min |
|     |               |         | URBarto2 | CTTCTCTTC ACAATTTCAT  |                    |      |                                               |
|     | Secondary     | *B. henselae* | URBhen-f | TTGCTTTCA AAAAGCTT ATCAA     | 240 bp             | 16   |                                               |
|     |               |         | URBhen-r | CAAAAGAG GGATTACA AAATC       |                    |      | 95°C, 5 min; 35 cycles of 45 sec at 95°C, 45 sec at 50°C, 1 min at 72°C; 72°C, 7 min |
|     |               |         | URBcla-f | ATGCTAAAA GTGCTATA TTGG        | 285 bp             | 16   |                                               |
|     | Secondary     | *B. clarridgeiae* | URBcla-f | CCTCACACT AAAATATAA AAAAC |                    |      |                                               |

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