Molecular epidemiology and risk factors of Anaplasma spp., Babesia spp. and Theileria spp. infection in cattle in Chongqing, China

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Abstract

Tick-borne pathogens (TBPs) seriously affect cattle production and can be economically damaging. The epidemiology of these organisms in the Chongqing municipality of China is not well described. This study aimed to investigate the prevalence and risk factors of TBPs including Anaplasma spp., Babesia spp. and Theileria spp. in cattle in Chongqing municipality. The results showed that 43.48% (150/345) of cattle were infected with at least one TBP, of which single infections were detected in 104 (30.14%), double infections in 34 cattle (9.86%) and triple infections in 12 (3.48%) of the cattle. The overall prevalence of Anaplasma spp., Theileria spp. and B. bigemina were 22.32%, 23.19% and 7.24%, respectively. Among these, the prevalence of A. bovis, A. central, A. phagocytophilum, A. platys, A. marginale, T. sinensis and T. orientalis were 8.41%, 7.83%, 4.93%, 4.35%, 2.61%, 22.32% and 2.60%, respectively. We could not detect B. bovis, T. annulata, T. luwenshuni or T. ulen-bergi in cattle. Cattle ≥1-year-old were more likely to be infected with Theileria spp. [adjusted odd ratio (AOR) = 2.70, 95% CI = 1.12–6.56] compared with younger cattle, while cattle ≥1-year-old had reduced susceptibility to B. bigemina (AOR = 0.14, 95% CI = 0.03–0.60). Cattle living at higher altitude (≥500 m) were more susceptible to B. bigemina (AOR = 6.97, 95% CI = 2.08–23.35) and Theileria spp. infection (AOR = 1.87, 95% CI = 1.06–3.32). The prevalence of Theileria spp. on farms with cats was significantly higher than that without cats (AOR = 2.56, 95% CI = 1.12–5.88). Infection with A. bovis and A. central were significantly associated with A. phagocytophilum infection. Furthermore, there were significant associations between A. bovis and A. central infection, T. sinensis and A. marginale infection, and B. bigemina and T. orientalis infection. This study provides new data on the prevalence of Anaplasma spp., Babesia spp. and Theileria spp. in cattle in Chongqing, and for the first time we reveal a possible relationship between the afore-mentioned pathogens, which will help in formulating appropriate control strategies for these pathogens in this area.
Introduction

Tick-borne pathogens (TBPs) have always attracted the attention of researchers, not only for their damaging influence upon livestock production but also for their public health threat [1]. Among the tick-borne diseases, anaplasmosis, babesiosis and theileriosis are the most important and are distributed widely. These organisms affect cattle worldwide [2]. Till now, five Anaplasma pathogens (A. marginale, A. bovis, A. centrale, A. phagocytophilum, and A. platys) have been reported to cause bovine anaplasmosis, of which A. phagocytophilum has been shown to infect a variety of animals and humans [3–5]. Two mainly Babesia pathogens, Babesia bovis and B. bigemina, were found responsible for bovine babesiosis [6], and three species of Theileria including T. annulata, T. sinensis, and T. orientalis (also named T. sergenti) were the main causative agents of bovine theileriosis [7–10], and recently, T. luwenshuni has also been detected in blood samples from cattle and yaks [9].

Numerous studies have reported the infection and prevalence of Anaplasma spp., Babesia spp. and Theileria spp. in cattle across many countries [4,5,11–16]. In China, there have also been many studies [9,17–22]. However, these studies usually focus on single pathogen infections, and records on pathogen co-infections, the risk factors, and the mutual influence of each pathogen in cattle are absent. In addition, studies relating to the aforementioned pathogens in cattle in China have mainly been restricted to the northwest region, while the information is very limited for southwest China.

The total number of cattle approximated 300 million at the end of 2015 in Chongqing, and is one of the economic pillars of animal husbandry in this city. However, the prevalence of Anaplasma spp., Babesia spp., and Theileria spp. in cattle in this area is unclear. The objectives of this study were 1) to detect Anaplasma spp., Babesia spp., and Theileria spp. in cattle in Chongqing, 2) to analyze the risk factors for infection of Anaplasma spp., Babesia spp., and Theileria spp. in cattle in Chongqing, 3) to evaluate the associations of the aforementioned pathogens in cattle in Chongqing.

Materials and methods

Study area

Chongqing municipality is located in the southwest of China, between the northern latitudes of 28.10°–32.13°, and eastern longitudes of 105.11°–110.11°. Its altitude ranges between 73.1 m at the Yangtze River in Wushan and 2796.8 m at Liangshan peak in Wuxi. The climate tends to be subtropical, with a monsoon/humid climate and has an average annual temperature of 16–18°C.

Blood sample collection and DNA extraction

Three hundred and forty five sodium citrate anticoagulated blood samples were collected from 10 ranches located in Tongnan, Rongchang, Jiangjing, Changshou, Liangping, Kaizhou, Yunyang, Wushan, Fuling, and Qianjiang, from May 2016 to April 2017. The ranches were selected based on the number of cattle (≥50) and convenience of sampling. The sampled animals were randomly selected from apparently healthy cattle, and the information including gender and age of cattle, as well as the altitude and the existence of cats in ranches were recorded. The blood samples were sent back to the laboratory within an ice box. Whole blood genome was extracted using a Wizard Genomic extraction kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. This study was approved by the Ethics Committee of Southwest. Consent was obtained from cattle owners before the collection of blood samples from their cattle by an experienced, practicing veterinarian.
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PCR detection of *Anaplasma spp.*, *Babesia* spp., and *Theileria* spp.

*Anaplasma* spp. (*A. bovis, A. central, A. marginale, A. phagocytophilum and A. platys*), *Babesia* spp. (*B. bovis* and *B. bigemina*) and *Theileria* spp. (*T. annulata, T. sinensis, T. orientalis, T. luwenshuni* and *T. uilenbergi*) infections were detected by PCR or nested PCR using the primers reported in previous studies [7,23–29], the detail of primers can be found in S1 Table. The primers were synthesized by Bioligo Biotechnology Co., Ltd (Shanghai, China). The PCRs were performed according to the amplification programs in Table 1, with a volume of 12.5 μL in the reaction system including: 6.25 μL Premix Taq (containing TaKaRa Taq, dNTP Mixture and Taq Buffer) (Takara Dalian, China), 0.5 μL of each forward and reverse primer (20 μmol/L), 1 μL whole blood genome and 4.25 μL ddH₂O. The amplified PCR products were photographed after electrophoresis in 1% agarose gels. The PCR amplification product were randomly selected for sequencing to verify the reliability of test.

Risk factor analysis

Multivariable logistic regression was performed in SPSS for Windows (18.0 version, SPSS Inc., Chicago, IL, USA) to analyze factors associated with aforementioned infections. Adjusted odd ratios (AOR) and 95% confidence intervals (CI) were calculated. A p-value of <0.05 was considered statistical significant.

Results

Prevalence of *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. infection

A total of 345 cattle in Chongqing were included in this study. Detailed information pertaining to infection is shown in Table 1 and Fig 1. The results showed that 43.48% (150/345) of cattle were infected with at least one TBP, of which single infections were detected in 104 (30.14%), double infections in 34 cattle (9.86%) and triple infections in 12 (3.48%) of the cattle. The overall prevalence of *Anaplasma* spp., *Theileria* spp., and *B. bigemina* in cattle were 22.32% (77/345), 23.19% (83/345), and 7.24% (25/345), respectively. Among the *Anaplasma* spp. detected, *A. bovis* (29/345, 8.41%) was the most prevalent species recorded, followed by *A. centroplus* (17/345, 4.93%), and *A. platys* (15/345, 4.35%), while infection with *A. marginale* (9/345, 2.61%) was the lowest. Among the *Theileria* spp., *T. sinensis*...
and *T. orientalis* infections in cattle were 22.32% (77/345) and 2.60% (9/345), respectively. In addition, we could not detect *B. bovis*, *T. annulata*, *T. luwenshuni*, or *T. uilenbergi* in this study.

### Risk factor analysis based on blood sample data

The prevalence of *Anaplasma* spp. (25.13%) and *Theileria* spp. (27.27%) in male cattle was significantly higher than that in females (*Anaplasma* spp.: AOR = 2.18, 95% CI = 1.05–4.52; *Theileria* spp.: AOR = 3.27, 95% CI = 1.47–7.25). Cattle ≥ 1-year were more likely to be infected with *Theileria* spp. than cattle aged below 1-year of age (25.00% vs. 20.99%), and the difference was statistically significant (AOR = 2.70, 95% CI = 1.12–6.56). In contrast, cattle ≥1-year had a lower risk of *B. bigemina* infection (AOR = 0.14, 95% CI = 0.03–0.60). Ranches at an altitude ≥500 m was found to be a risk factor for *B. bigemina* (AOR = 6.97, 95% CI = 2.08–23.35) and *Theileria* spp. infection (AOR = 1.87, 95% CI = 1.06–3.32). With exception of *Theileria* spp. (AOR = 2.56, 95% CI = 1.12–5.88), there were no significant associations between presence of cats and infection with *Anaplasma* spp., or *B. bigemina*, (Table 2).

### Risk factor analysis based on pathogen co-infection

In order to evaluate the effect of specific pathogen infections and how they influence other pathogen infections within the same host, we considered each tested pathogen species as a potential risk factor in the analysis. The results of correlation analyses between each species of pathogen in infected cattle in Chongqing, are shown in Table 3. Infection with *A. bovis* and *A. central* were significantly associated with *A. phagocytophilum* infection, and *A.
phagocytophilum was more likely to increase the risk of *A. central* infection (AOR = 3.80, 95% CI = 1.10–13.18, *p* = 0.035). However, *A. central* was less likely to impact upon infection with *A. phagocytophilum* (AOR = 3.50, 95% CI = 0.97–12.59, *p* = 0.055). Furthermore, there was a significant association between *A. bovis* and *A. central* infection, *T. sinensisi* and *A. marginale*

### Table 2. Multivariate analysis of selected factors and their association with *Anaplasma* spp., *B. bigemina* and *Theileria* spp. infection in cattle in Chongqing of southwest China.

| Factors          | Positive/Examined | Prevalence (%) | AOR (95% CI)     | *p*-value |
|------------------|-------------------|----------------|------------------|-----------|
| **Anaplasma spp.** |                   |                |                  |           |
| Gender           |                   |                |                  |           |
| Male             | 47/187            | 25.13          | 2.18 (1.05–4.52) | 0.037     |
| Female           | 30/158            | 18.99          | Reference        |           |
| Age              |                   |                |                  |           |
| ≥ 1 year         | 58/264            | 21.97          | 1.68 (0.75–3.75) | 0.204     |
| < 1 year         | 19/81             | 23.46          | Reference        |           |
| Altitude         |                   |                |                  |           |
| ≥ 500 m          | 31/156            | 19.87          | 0.62 (0.34–1.10) | 0.104     |
| < 500 m          | 46/189            | 24.34          | Reference        |           |
| Cats presence    |                   |                |                  |           |
| Yes              | 46/204            | 22.55          | 1.87 (0.89–3.93) | 0.100     |
| No               | 31/141            | 21.99          | Reference        |           |
| **B. bigemina**  |                   |                |                  |           |
| Gender           |                   |                |                  |           |
| Male             | 14/187            | 7.49           | 0.450 (0.10–1.92) | 0.280     |
| Female           | 11/158            | 6.69           | Reference        |           |
| Age              |                   |                |                  |           |
| ≥ 1 year         | 13/264            | 4.92           | 0.14 (0.03–0.60) | 0.009     |
| < 1 year         | 12/81             | 14.81          | Reference        |           |
| Altitude         |                   |                |                  |           |
| ≥ 500 m          | 20/156            | 12.82          | 6.97 (2.08–23.35) | 0.002     |
| < 500 m          | 5/189             | 2.65           | Reference        |           |
| Cats presence    |                   |                |                  |           |
| Yes              | 22/204            | 10.78          | 1.40 (0.28–7.03) | 0.681     |
| No               | 3/141             | 2.13           | Reference        |           |
| **Theileria spp.** |                 |                |                  |           |
| Gender           |                   |                |                  |           |
| Male             | 51/187            | 27.27          | 3.27 (1.47–7.25) | 0.004     |
| Female           | 32/158            | 21.52          | Reference        |           |
| Age              |                   |                |                  |           |
| ≥ 1 year         | 66/264            | 25.00          | 2.70 (1.12–6.56) | 0.027     |
| < 1 year         | 17/81             | 20.99          | Reference        |           |
| Altitude         |                   |                |                  |           |
| ≥ 500 m          | 52/156            | 33.33          | 1.87 (1.06–3.32) | 0.031     |
| < 500 m          | 31/189            | 16.40          | Reference        |           |
| Cats presence    |                   |                |                  |           |
| Yes              | 55/204            | 26.96          | 2.56 (1.12–5.88) | 0.025     |
| No               | 28/141            | 19.86          | Reference        |           |

Note: AOR = adjusted odds ratio.

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infection, and *B. bigemina* and *T. orientalis* infection (*p*<0.05). There were no significant associations between other pathogens that we aimed to identify.

**Discussion**

For the first time, this systematic study investigated the epidemiology of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. infection in cattle in Chongqing, China. The infection rate of *Anaplasma* spp. in our study was lower than that reported in Algeria [5] and in Tunisia [16], but higher than that reported in northwest China [30]. The prevalence of *A. bovis* (8.41%) in cattle in Chongqing was higher than that of cattle reported in other locations, where the prevalence varied from 3.9% to 6.2% [5,16,18,30]. In contrast, the prevalence of *A. centrale* (7.83%) was lower than that of cattle in previous studies (range between 12.1%-39.4%) [5,11,16,31,32]. Compared to the high prevalence of *A. marginale* in cattle in Madagascar (89.7%), north-eastern Uganda (82.9%) [11], South Africa (57%) [31], Thailand (39.1%) [13] and in China (31.6%) [33], we demonstrated a relatively low infection rate of *A. marginale* (2.61%) in Chongqing. In addition, 4.93% of cattle tested positive for *A. phagocytophilum* in this study, which was similar to the positivity rate (5.3%) of this pathogen in white yaks [30]. *A. platys* infection
in cattle was first reported in Algeria [4], while Ben et al. reported a prevalence of A. platys-like species (3.5%, 13/367) in cattle in Tunisia [34]. In this study, we noted a prevalence of A. platys (4.35%, 15/345) in cattle for the first time in Chongqing.

The prevalence of B. bigemina in this study was similar to previous research by Liu et al [21], and is lower than that reported in other provinces of China [19–21,35], South Africa [36], and in Tanzania [37]. However, the prevalence in our study was higher than that recorded in the Philippines [38]. In this survey, only T. sinensis and T. orientalis were detected, with the prevalence being lower than T. sinensis and T. orientalis infection rates recorded elsewhere [13,14,17]. Similar to the previous report [17], we did not detected B. bovis infection in cattle. The reason may be that 1) B. bovis infection in tick is usually lower than B. bigemina, which result a lower transmission rates of B. bovis, and 2) B. bovis-infected red blood cells usually accumulate in the capillary bed and leading to low parasitemia in circulating blood [17]. For the reasons that T. annulata, transmitted by Hyalomma anatolicum anatolicum, is mainly distributed in Northern China [39], T. luwenshuni and T. uilenbergi, both transmitted by Haemaphysalis qinghaiensis and H. longicornis, usually infected sheep and goats in China [27], and there is no evidence of above ticks existence in Chongqing. It was not strange that we did not detected T. annulata, T. luwenshuni, or T. uilenbergi infection in cattle from Chongqing.

There were 117 described species in the Chinese tick, 38 of which carry multiple pathogens [40], and most of the ticks including H. anatolicum, H. qinghaiensis, H. longicornis, H. bispinosa, Rhipicephalus (Boophilus) microplus, R. sanguineus, Dermacentor abanesis, D. silvarum and D. nuttalli were founded in northwest, northeast or central of China [39,41-44], and these ticks are responsible for transmission of a large amount of TBPs. However, the only reported tick specie in Chongqing was R. microplus [45], which was recorded to be the vector of A. phagocytophilum, A. marginale, B. bigemina and B. bovis in China [40,46,47]. The differences in the prevalence of some parasites in this study compared to that reported previously in other studies in China or other countries, might be associated with geographical difference and variation in tick species.

Risk factor analysis revealed a significant correlation of altitude and age with the prevalence of B. bigemina and Theileria spp., which supported a previous report that there was a trend in increased seropositivity for B. bigemina infection with age [37]. In addition, gender is a risk factor associated with prevalence of Anaplasma spp. and Theileria spp., in cattle, which showed that male cattle had higher risk for these two type of pathogens infection, and the presence of cats in farm had positive effect on Theileria spp. infection in cattle from Chongqing, and the reasons for these phenomenon are not clear.

This study first took a single infection as a risk factor in evaluating the impact on infection with other pathogens. We found that cattle infected with A. bovis or A. central were more likely to be infected with A. phagocytophilum, and there was also a strong association between A. bovis and A. central infection. In addition, a very close relationship was observed for co-infection with T. sinensis and A. marginale, and B. bigemina and T. orientalis. Anaplasma spp., Babesia spp., and Theileria spp. are all tick borne pathogens (TBPs), and some ticks can harbor mixed TBPs [40,47,48]. For the reasons that one species of TB can be spread by different types of ticks, and equally that the same type of tick may also be the transmission vector for many species of TBPs, the significant correlation of the aforementioned pathogens might be attributed to the fact that infected cattle were bitten by ticks carrying different pathogens. From current data, it is not possible to estimate the chronological order of the aforementioned pathogen infections but there does appear to be significant relationships among some of these pathogens during infection of cattle. Parasite-parasite interaction may modify the impact of the pathogenic species and affect the performance and survival of host [49,50]. It is a pity that this study failed to evaluated the effect of above TBPs on health of cattle, since all the sampled
animals in this study were apparently healthy, and we did not track the outcome of these cattle and the causes of their death. Further research should be conducted to elucidate the type of ticks present in Chongqing and the proportion of ticks that carry TBP. Furthermore, attempts should be made to confirm whether a pathogen significantly increases the incident infection of other pathogens and the effects on production performance of cattle.

Conclusions
The results of the present survey indicated that infection of cattle with Anaplasma spp., Babesia spp., and Theileria spp. is widespread in Chongqing. We provide a possible relationship between afore-mentioned pathogenic infections, which will help in formulating appropriate control strategies for these pathogens in this area.

Supporting information
S1 Table. Primers used for Anaplasma spp., Babesia spp. and Theileria spp. detection in cattle. (DOCX)

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