Prevalence of *Anaplasma phagocytophilum* in ruminants, rodents and ticks in Gansu, north-western China

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The zoonotic rickettsial pathogen *Anaplasma phagocytophilum* has a broad geographical distribution and a high degree of biological and clinical diversity. To determine the prevalence of *Anaplasma phagocytophilum* in the Gannan Tibetan Autonomous Prefecture of Gansu Province, north-western China, four ruminant species, one rodent and one tick species were examined for *Anaplasma phagocytophilum* infection. DNA from *Anaplasma phagocytophilum* was detected by nested PCR in blood samples from 21/49 sheep (42.9 %), 35/91 goats (38.5 %), 51/158 yaks (32.3 %) and 7/20 cattle–yaks (35.0 %), and in spleen samples from 2/12 rodents (16.7 %). For samples from tick larvae and nymphs, 105 pools were tested; one of 46 larval tick pools was positive and seven of 59 nymphal tick pools were positive. For adult ticks, 40/598 female ticks (6.7 %) and 26/528 male ticks (4.9 %) were positive. The prevalence of *Anaplasma phagocytophilum* in female ticks was higher than that in males, although the difference was not statistically significant \((P > 0.05)\). Sequences analysis based on the 16S rRNA gene indicated that the strains in the study area were distinct from previously reported *Anaplasma phagocytophilum* in other continents. These results add new information on the epidemiology of *Anaplasma phagocytophilum* and indicate the tick–animal cycle of anaplasmosis in the area. To the best of our knowledge, this is the first report of *Anaplasma phagocytophilum* infection in Gansu Province in north-western China.

INTRODUCTION

Human granulocytic anaplasmosis [HGA; formerly known as human granulocytic ehrlichiosis (HGE)] is an emerging tick-borne disease caused by the obligate intracellular bacterium *Anaplasma phagocytophilum* (formerly known as *Ehrlichia phagocytophila*, *Ehrlichia equi* or the HGE agent). Since the first case was reported in the USA in 1990, HGA has been described in both Europe and Asia (Chen et al., 1994; Dumler et al., 2005; Ohashi et al., 2005). In China, the first case of HGA was reported in Anhui Province in 2006 (Zhang et al., 2008). *Anaplasma phagocytophilum* is thought to be maintained naturally in small mammal–tick cycles, with *Ixodes* ticks as vectors (Bown et al., 2003; Dumler et al., 2001). A wide range of mammals serve as reservoir hosts in the natural life cycle of *Anaplasma phagocytophilum* (Jin et al., 2012).

To date, few studies have been conducted to investigate the prevalence of *Anaplasma phagocytophilum* in livestock, rodents and ticks in China. *Anaplasma phagocytophilum* infection has been found in sheep, goats, cattle, rabbits, nine species of rodents and four species of ticks in Zhejiang Province (south-eastern China) and Heilongjiang and Jilin Provinces (north-eastern China) (Chahan et al., 2005; Zhan et al., 2009a, b, 2010a, b). However, systematic surveys of mammals and ticks in north-western China have not been reported. Furthermore, information is scarce on the animal reservoirs of *Anaplasma phagocytophilum* in China. The objective of this study was to investigate the presence of *Anaplasma phagocytophilum* infection in multiple hosts in the Gannan Tibetan Autonomous Prefecture in north-western China.

METHODS

Study site and collection of specimens. Gannan Tibetan Autonomous Prefecture includes seven counties, Lintan, Zhuoni, Luqu, Maqu, Diebu, Zhouqu and Xiahe, and is situated at the east edge of the Qinghai–Tibetan Plateau. It can be divided into three
parts: the southern mountainous region with a moderate climate, an important forest zone in Gansu; the eastern hilly area; and the eastern meadows and grassland region. EDTA–K$^+$ and anticoagulated blood samples were collected from goats, sheep, yaks and cattle–yaks (domestic cattle crossed with yaks, found mainly on the Qinghai–Tibetan Plateau) in June 2010 from Xiahe, Lintan, Luqu and Zhourui counties in the Gannan Tibetan Autonomous Prefecture in Gansu Province, north-western China, as shown in Fig. 1. These animals were infested with ticks and showed signs of depression, thin hair and weight loss. Twelve striped field mice (Apodemus agrarius) were captured from agrarian farms near the mountains in Lintan county. Each rodent was euthanized and dissected. The spleen was removed and ground with sterilized normal PBS. A total of 1881 questing Haemaphysalis longiarensis ticks were collected between March and May 2011 by flagging the undergrowth with a flannel cloth. Ethical approval was given by Lanzhou Veterinary Research Institute Ethics Committee.

**DNA extraction.** Tick larvae and nymphs were pooled before DNA extraction; each tick pool consisted of ten larvae, five nymphs or a single adult tick. Tick specimens were soaked for 15 min in 70 % ethanol and washed twice in sterile, distilled water. The tick specimens were then dissected into pieces in 50 µl PBS and placed in a separate 1.5 ml Eppendorf tube. The samples were incubated with 20 µl (>600 mAU ml$^{-1}$) proteinase K for 2 h at 56 °C and then boiled at 100 °C for 10 min to inactivate the proteinase K. The homogenate was centrifuged at 8000 $\times$ g for 10 min at room temperature and the supernatant was processed further. DNA was extracted from the ruminant blood samples, rodent spleens and ticks using a Gentra Puregene DNA Purification kit (Qiagen) according to the manufacturer’s instructions.

**PCR amplifications.** The extracted DNA was examined for the presence of *Anaplasma phagocytophilum* by nested PCR for amplification of the 16S rRNA gene using specific primers (first-round primers: EE1f, 5’-AGTCACTGACCCAACCTTAAATGGCTG-3’; nested primers: EE3f, 5’-GTCGAACGGATTATTCTTTATAGCTTGC-3’, and EE3r, 5’-CCCTTCCGTGTAAGAAGGATCTAATCTCC-3’), and EE4r, 5’-CCCTTCCGTGTAAGAAGGATCTAATCTCC-3’), which amplified a 925 bp fragment, as described by Barlough et al. (1996).

Positive samples were confirmed by PCR using another pair of nested primers (SSAP2f, 5’-GCTGAATGTGGGGATAATTTAT-3’, and SSAP2r, 5’-ATGGCTGCTTCCTTTCGGTTA-3’), amplifying a 641 bp fragment as described by Kawahara et al. (2006). The reaction was performed in an automatic thermocycler (Bio-Rad) in a total volume of 25 µl, containing 2.5 µl 10 × PCR buffer (Mg$^{2+}$ Plus; TaKaRa), 2.0 µl dNTPs (2.5 mM), 0.25 µl (1.25 U) Taq DNA polymerase (TaKaRa), 2.0 µl template DNA, 1.0 µl each primer (10 pmol) and 16.25 µl distilled water. Genomic DNA extracted from *Anaplasma phagocytophilum*-positive tick samples that had been verified by sequencing was used as the positive control, and sterile water was used as the blank control. The cycling conditions for the amplification comprised 4 min of denaturation at 94 °C, 35 cycles of 94 °C for 1 min, annealing for 1 min at 55 °C and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. The PCR products were visualized by UV transillumination in a 1.5 % agarose gel following electrophoresis and staining with ethidium bromide.

**Sequencing and phylogenetic analyses.** The 925 bp nested PCR products of the 16S rRNA gene of *Anaplasma phagocytophilum* were purified using a TaKaRa Agarose Gel DNA purification kit version 2.0, ligated into a pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* JM109 competent cells. Recombinant clones were selected for sequencing using BigDye Terminator Mix (Sangon). The sequences obtained were compared with previously published sequences deposited in GenBank using BLAST. A phylogenetic tree was then constructed based on the sequence distance method using the neighbour-joining algorithm with the Kimura two-parameter model of the MEGA4.0 software (Tamura et al., 2007) (Fig. 2).

**Statistical analysis.** The results were analysed using a $\chi^2$ test in Predictive for Analytics Software (PASW) Statistics 18. A difference was considered statistically significant at $P<0.05$.

**RESULTS AND DISCUSSION**

*Anaplasma phagocytophilum* has been recognized as an animal pathogen and is an emerging human pathogen of public health relevance (Chen et al., 1994). In addition to humans, many domestic animals, such as dogs, cats,

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**Fig. 1.** Sample collection sites in Gannan Tibetan Autonomous Prefecture of Gansu Province, north-western China. Grey shading indicates the areas where *Anaplasma phagocytophilum* was detected previously in rodents and ticks. The study site for the current study is also indicated.

**Fig. 2.** Phylogenetic tree based on the 16S rRNA gene sequences of *Anaplasma phagocytophilum* (Ap). Bar, 0.005 nucleotide substitutions per site.
In this study, *Anaplasma phagocytophilum* was found in all four ruminant species tested. Similar levels of prevalence of *Anaplasma phagocytophilum* were found in the blood samples from sheep (21/49, 42.9%), goats (35/91, 38.5%), yaks (51/158, 32.3%) and cattle–yaks (7/20, 35.0%) (Table 1). *Anaplasma phagocytophilum* infection has been reported in sheep, goats, cattle and rabbits previously in other parts of China (Chahan et al., 2005; Zhan et al., 2009b, 2010a, b). The results of this study confirmed that sheep and goats in the Gannan Tibetan Autonomous Prefecture were also infected by *Anaplasma phagocytophilum*. Moreover, yaks and cattle–yaks were also found to be infected by the pathogen. These results thus add new information on the reservoirs of this disease agent.

In Asia, *Apodemus agrarius* is considered to be the most important reservoir of *Anaplasma phagocytophilum* (Jin et al., 2012). Three strains of *Anaplasma phagocytophilum* have been isolated from *Apodemus agrarius* and *Tscherskia triton* rodents in China (Zhan et al., 2010a). *Anaplasma phagocytophilum* infection has been found in *Apodemus agrarius*, *Apodemus peninsulae*, *Apodemus sylvaticus*, *Clethrionomys rufocanus*, *Niviventer confucianus*, *Rattus losea*, *Rattus norvegicus* and *Tamias sibiricus* collected in Heilongjiang, Jilin and Zhejiang Provinces, with a mean prevalence of 6.8% (38/557) (Zhan et al., 2009a). Previous reports suggest that wild rodents may act as competent reservoirs of *Anaplasma phagocytophilum* (Jin et al., 2012). In our study, *Apodemus agrarius* was the only rodent species trapped in Lintan county, and 2/12 (16.7%) of the trapped individuals were found to be infected with *Anaplasma phagocytophilum* (Table 1). The prevalence of *Anaplasma phagocytophilum* in *Apodemus agrarius* in the present study is consistent with the mean prevalence of 14.7% found in the same rodent species collected in Jilin Province (5/24, 20.8%, Cao et al., 2006; 10/78, 12.8%, Zhan et al., 2010b). These data suggest that *Apodemus agrarius* may contribute to the maintenance of a natural cycle of *Anaplasma phagocytophilum* in China. *Apodemus agrarius* is distributed over an extensive area in mainland China and is also known to be a major reservoir host for *B. burgdorferi* sensu lato (Chu et al., 2008). Our results further demonstrate the role of *Apodemus agrarius* as a reservoir of *Anaplasma phagocytophilum* in the Gannan Tibetan Autonomous Prefecture in north-western China.

*Anaplasma phagocytophilum* is usually associated with ticks of the genus *Ixodes*, including *Ixodes scapularis*, *Ixodes pacificus* (Parola et al., 2005) and *Ixodes dentatus* (Goethert & Telford, 2003) in the USA; *Ixodes ricinus* and *Ixodes trianguliceps* in Europe (Bown et al., 2008); and *Ixodes persulcatus* in Asia (Cao et al., 2003). However, because of its capacity for trans-stadial and transovarial transmission, *Dermacentor albipictus* may be another vector of *Anaplasma phagocytophilum* (Baldridge et al., 2009). Moreover, *Anaplasma phagocytophilum* DNA has been found in *Haemaphysalis concinna*, *Haemaphysalis longicornis*, *I. persulcatus* and *Dermacentor silvarum* in China (Cao et al., 2003; Jiang et al., 2011). These results indicate that numerous tick species may maintain or transmit *Anaplasma phagocytophilum*. More than 109 tick species have been recorded in China (Yin & Luo, 2007). Several molecular epidemiological surveys have been conducted to investigate the presence of pathogenic *Anaplasma phagocytophilum* in ticks (Bown et al., 2008; Cao et al., 2006). However, little is known about the prevalence of *Anaplasma phagocytophilum* in *H. qinghaiensis*, which is distributed widely throughout Gannan Tibetan Autonomous Prefecture. In the current study, of the 105 tick pools tested, only 1/46 larval tick pools (2.2%) was positive for *Anaplasma phagocytophilum*, whereas 7/59 nymphal tick pools (11.9%) were positive (Table 2). In total, 40/598 female ticks (6.7%) and 26/528 male ticks (4.9%) were infected with *Anaplasma phagocytophilum*. The prevalence of *Anaplasma phagocytophilum* infection was found to be significantly higher in females than in males in *I. scapularis* (84.9 and 9.4%, respectively) in the USA (Massung et al., 2003).

### Table 1. Prevalence of *Anaplasma phagocytophilum* in Gannan Tibetan Autonomous Prefecture

<table>
<thead>
<tr>
<th>Location</th>
<th>Sheep</th>
<th>Goat</th>
<th>Yak</th>
<th>Cattle–yak</th>
<th>Apodemus agrarius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiahe</td>
<td>11/20 (55.0)</td>
<td>16/26 (61.5)</td>
<td>–</td>
<td>3/10 (30.0)</td>
<td>–</td>
</tr>
<tr>
<td>Lintan</td>
<td>7/10 (70.0)</td>
<td>8/36 (22.2)</td>
<td>19/36 (52.8)</td>
<td>–</td>
<td>2/12 (16.7)</td>
</tr>
<tr>
<td>Luqu</td>
<td>3/19 (15.8)</td>
<td>10/24 (41.7)</td>
<td>22/81 (27.2)</td>
<td>4/10 (40.0)</td>
<td>–</td>
</tr>
<tr>
<td>Zhuoni</td>
<td>–</td>
<td>1/5 (20.0)</td>
<td>10/41 (24.4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>21/49 (42.9)</td>
<td>35/91 (38.5)</td>
<td>51/158 (32.3)</td>
<td>7/20 (35.0)</td>
<td>2/12 (16.7)</td>
</tr>
</tbody>
</table>

–, No sample collection.
Prevalence of *Anaplasma phagocytophilum* in *H. qinghaiensis* ticks

<table>
<thead>
<tr>
<th>Tick life stage/ gender</th>
<th>Tick pools (no. positive/ no. tested)</th>
<th>Prevalence of infection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larva</td>
<td>1/46</td>
<td>2.2</td>
</tr>
<tr>
<td>Nymph</td>
<td>7/59</td>
<td>11.9</td>
</tr>
<tr>
<td>Female</td>
<td>40/598</td>
<td>6.7</td>
</tr>
<tr>
<td>Male</td>
<td>26/528</td>
<td>4.9</td>
</tr>
<tr>
<td>Total</td>
<td>74/1231</td>
<td>6.0</td>
</tr>
</tbody>
</table>

2005) and in *I. ricinus* (47.6 and 8.6 %, respectively) in Poland (Staniczak et al., 2004). In the present study, the prevalence of *Anaplasma phagocytophilum* in female ticks was also higher than that in male ticks, which was consistent with the previous reports, although the difference was not statistically significant (*P* > 0.05). On average, at least 6.0 % of tick samples (each sample comprised a pool of ten larvae, five nymphs or a single adult tick) was found to be infected. This study confirmed the existence of *Anaplasma phagocytophilum* in *H. qinghaiensis* in north-western China. Although this tick species was shown to harbour *Anaplasma phagocytophilum*, its capacity as a vector has yet to be validated through transmission studies.

Sequence analysis revealed that the partial 16S rRNA gene sequences amplified from these samples tested in this study were 100 % identical and varied from all known *Anaplasma phagocytophilum* sequences in GenBank, with 98.2–99.0 % nucleotide identity. The newly detected *Anaplasma phagocytophilum* sequences determined in this study (GenBank accession no. JX914659) were most similar to the sequences detected in deer from Japan (GenBank accession no. AB196720), possessing an 11 bp difference, but differed more compared with sequences detected in rodents from Zhejiang Province of south-eastern China (GenBank accession no. DQ458805) with 18 bp differences, and in ticks and rodents from Jilin Province of north-eastern China (GenBank accession nos DQ449948 and DQ342324) with 17 bp differences, as shown in Fig. 2. The phylogenetic tree based on alignments of the 16S rRNA gene sequence showed that the agent detected in this study was distant from the previously reported *Anaplasma phagocytophilum* in south-eastern and north-eastern China and in other places (Fig. 2).

In summary, we found that four ruminant species (sheep, goats, yaks and cattle–yaks), one rodent species (*Apodemus agrarius*) and one tick species (*H. qinghaiensis*) were infected with *Anaplasma phagocytophilum*. Sequence analysis based on the 16S rRNA gene indicated that the strains in the study area were distinct from previously reported *Anaplasma phagocytophilum* in other continents. To our knowledge, this is the first report of the existence of *Anaplasma phagocytophilum* in *H. qinghaiensis* ticks and in yaks and cattle–yaks. Since the first case of HGA was detected in Anhui Province in 2006, *Anaplasma phagocytophilum* infections have been found across Hubei, Henan, Anhui, Shandong, Helongjiang, Inner Mongolia, Xingjiang and Tianjin in China (Zhang et al., 2008). The results of our study comprise the first report of *Anaplasma phagocytophilum* infection in Gannan Tibetan Autonomous Prefecture in Gansu Province, China.

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