The first detection of *Anaplasma capra*, an emerging zoonotic *Anaplasma* sp., in erythrocytes

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ABSTRACT

An emerging infectious disease caused by "*Anaplasma capra*" was reported in a 2015 survey of 477 hospital patients with a tick-bite history in China. However, the morphological characteristics and parasitic location of this pathogen are still unclear, and the pathogen has not been officially classified as a member of the genus *Anaplasma*. *Anaplasma capra*-positive blood samples were collected, blood cells separated, and DNA of whole blood cells, erythrocytes, and leukocytes extracted. Multiplex PCR detection assay was used to detect whole blood cell, erythrocytes and leukocytes, DNA samples, and PCR identification, nucleic acid sequencing, and phylogenetic analyses based on *A. capra* groEL, 16S rRNA, gltA, and msp4 genes. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), Wright-Giemsa staining, chromogenic in situ hybridization (CISH), immunocytochemistry, and indirect immunofluorescence assay (IFA) were used to identify the location and morphological characteristics of *A. capra*. Multiple gene loci results demonstrated that erythrocyte DNA samples were *A. capra*-positive, while leukocyte DNA samples were *A. capra*-negative. Phylogenetic analysis showed that *A. capra* is in the same clade with the *A. capra* sequence reported previously. SEM and TEM showed one or more pathogens internally or on the outer surface of erythrocytes. Giemsa staining, CISH, immunocytochemistry, and IFA indicated that erythrocytes were *A. capra*-positive. This study is the first to identify the novel zoonotic tick-borne *Anaplasma* sp., "*Anaplasma capra*,” in host erythrocytes. Based on our results, we suggest revision of Genus *Anaplasma* and formally name “*A. capra*” as *Anaplasma capra* sp. nov.

KEYWORDS *Anaplasma capra*; zoonotic; erythrocyte; tick-borne; *Rickettsia*

Key points

- First identified the novel zoonotic tick-borne *Anaplasma* sp. in host erythrocytes.
- Suggest formally name the novel erythrocytes pathogen as *Anaplasma capra* sp. nov.

Introduction

In 2015, an emerging infectious disease caused by "*Anaplasma capra*,” was reported in a survey of 477 hospital patients with tick-bite history in Mudanjiang Forestry Central Hospital, Heilongjiang Province, China. A total of 28 (6%) of the 477 patients were diagnosed with "*A. capra*,” and the pathogen was isolated from three cases [1]. Patients positive for this pathogen presented with an undifferentiated influenza-like illness, gastrointestinal symptoms, rash, eschar, regional lymphadenopathy with potential progression to central nervous system involvement, and cerebrospinal fluid pleocytosis [1]. *Anaplasma capra* was provisionally nominated as a novel tick-borne zoonotic *Anaplasma* sp. by Li et al. (2015) [1] after being first identified in a goat (*Capra aegagrus hircus*) [2]. *Anaplasma capra* has been shown to infect humans, ruminants (sheep, goat, cattle, etc.), pet dogs, and wild animals (e.g. takin, musk deer, goral) [1,3–8], and has been detected in a variety of ticks, e.g. *Ixodes persulcatus*, *Haemaphysalis longicornis*, *H. concinna*, *H. qinghaiensis*, and *Rhipicephalus microplus* [9–12]. *Anaplasma capra* is distributed all over the world, including China, France, Japan, South Korea, and Italy [5,7,13–15], posing a potential health threat to both humans and animals.

The Genus *Anaplasma* comprises a group of Gram-negative obligate intracellular bacteria consisting of many members, e.g. *A. bovis*, *A. ovis*, *A. marginale*, etc.
A. centrale, and A. phagocytophilum [16]. While A. ovis, A. marginale, and A. centrale are known as intraerythrocytic pathogens, A. bovis and A. phagocytophilum infect monocytes and neutrophil granulocytes, respectively [17,18]. Anaplasma phagocytophilum presents as a mild to severe febrile illness, including multiple organ failure and death [1,19–21]. Anaplasma capra is a novel zoonotic Anaplasma sp., included with other Anaplasma species in the list of prokaryotic names with standing in nomenclature (LPSN), but not validly published. Its morphological characteristics and type cells infected are unclear. Although molecular detection and identification based on groEL, 16S rRNA, gltA, msp2, and msp4 genes of A. capra have been performed in the last five years [6], neither morulae nor other forms of the pathogen had been detected so far in peripheral blood smears [1,10]. Hence, studies to determine the host parasite site, morphological characteristics, and pathogenesis of A. capra is needed to distinguish it from other tick-borne Anaplasma infections.

Material and methods

Blood samples collection and preparation

Two goats with a history of tick bites were purchased from a goat farmer in Luoyang City, Henan province, central region of China. One goat was A. capra-positive by PCR, and the other was A. capra-negative. EDTA blood samples were collected from the two goats, and the erythrocytes and leukocytes separated from the blood samples using an Erythrocytes Separation Kit (TBD, Tianjin, China). The density gradient of the separation solution separates erythrocytes and leukocytes. Subsequently, DNA of the whole blood cells, erythrocytes and leukocytes, were extracted from the samples using a Blood DNA Kit (OMEGA, Norcross, GA, USA), according to the manufacturer’s recommendations. All DNA samples were stored at −20°C until used.

PCR, gene sequencing and phylogenetic analysis

A multiplex PCR detection assay was used to detect erythrocyte and leukocyte DNA samples targeting the A. capra groEL gene, as previously described [22] (Table 1). Anaplasma capra-positive samples were genetically profiled by amplification of the16S rRNA (rrs) gene as well as gltA and msp4 genes as previously described [1] (Table 1). Selected, A. marginale, A. platys, and A. centrale genes were used to exclude co-infections in A. capra-positive samples (Table 1). As positive controls, a co-infected DNA sample (concurrently infected with “A. capra,” A. bovis, A. ovis, and A. phagocytophilum) and A. marginale-
A. platys-, and A. centrale-positive samples were used, and negative control double distilled H₂O was used.

The PCR products were sequenced to confirm the presence of *Anaplasma capra* DNA. The sequences obtained (GenBank Accession Nos.: *groEL* MT804297, 16S rRNA MT799937, *gltA* MT804296, *msp4* MT804298) were compared with published sequences in GenBank using BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were performed and phylogenetic trees constructed based on the sequence distance method using the neighbor-joining (NJ) algorithm with Mega 7.0 software.

**Electron microscopy**

*Anaplasma capra*-positive erythrocytes were fixed with a commercial electron microscope fixing solution (Solarbio, Beijing, China). After fixation, the cells were removed by centrifugation. The fixed cells were 0.01 M phosphate buffer solution (PBS) buffer-washed, post-fixed with 2% OsO₄, water-washed, and dehydrated in graded ethanol (EtOH) series (30%, 50%, 70%, 80%, 90%, 100%, and 100% at 15-min intervals). For SEM, after the cells were dehydrated in graded EtOH series, the EtOH was replaced with isoamyl acetate, mounted on a metal stub, sputter-coated with gold for 5 min, and examined using a SU8100 electron microscope (HITACHI, Japan) at 3 kV. For TEM observation, the cells were in ultramicrotome (HITACHI, Japan) [23]. The morulae of *A. capra*, following lysis of the erythrocytes and centrifuged at 12,000 × g, were observed by TEM.

**Wright–Giemsa staining, CISH, immunocytochemistry, and IFA**

Blood cell smears were fixed in methanol for 10 min and stained with Wright–Giemsa stain, and the intracellular morulae observed under light microscopy. To identify the specificity of the intracellular morulae detected in the blood cells, CISH, immunocytochemistry, and IFA were conducted with *A. capra*-positive samples.

CISH was performed with a commercial kit according to the manufacturer’s protocol (Invitrogen, Carlsbad, USA). Briefly, the blood films were reverse-stained with eosin, visualized using Digital Slice Scanner (Pannoramic MIDI, Hungary), and fixed with a fixative (Solarbio, Beijing, China). The CISH probes were designed specifically for *Anaplasma capra* (Invitrogen, Carlsbad, USA). To achieve sufficient signal to background ratio, multiple probes were targeted along each individual lncRNA/mRNA sequence of the *A. capra* sp. nov *groEL* gene (KM206275). A set of 16 probes covering the entire length of the mRNA molecule allowed for optimal signal strength. The probes were labeled with digoxigenin (DIG), and brown-stained cells were considered positive. For immunocytochemistry, the *A. capra*-positive serum samples collected from the goat were used as the primary antibody and incubated at 4°C overnight. Subsequently, HRP-conjugated Rabbit Anti-Goat IgG (H+L) (Servicebio, Wuhan, China) was applied to the smears and incubated at 37°C for 1 h. The antibody was visualized using 3,3′-diaminobenzidine (Servicebio, Wuhan, China), and the images recorded using a light microscope (Nikon, Tokyo, Japan). As negative controls, the antiserum from the *A. capra*-negative goat and *A. capra*-negative blood smears were processed in the same manner and examined.

For IFA, *A. capra*-positive whole blood cells were processed for the preparation of antigen slides. The *A. capra*-positive serum samples collected from goats were used as the primary antibody, and Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific, Carlsbad, USA) used as a secondary antibody [20]. The *A. capra*-negative whole blood cells smear were used as negative controls. The slides were examined using a Digital Slice Scanner (Pannoramic MIDI, Hungary) and cells with blue fluorescence considered positive.

**Results**

The multiplex PCR detection assay showed that the whole blood DNA samples were positive for “*A. capra,*” *A. bovis,* and *A. phagocytophilum.* Three erythrocyte DNA samples were *A. capra*-positive only, while three leukocytes DNA samples were both *A. bovis-* and *A. phagocytophilum*-positive (Figure 1). Therefore, multi-site identification was conducted based on 16S rRNA, *gltA,* and *msp4* genes of *A. capra* using erythrocyte and leucocyte DNA samples (Figure 2). The PCR identification results based on multiple gene loci showed that all erythrocyte DNA samples were *A. capra*-positive and *A. marginale*-, *A. platys*-, and *A. centrale*-negative; leucocyte DNA samples were *A. capra*-negative; and samples infected with *A. bovis,* *A. ovis,* *A. marginale,* *A. platys,* and *A. centrale* were *A. capra*-negative.

Subsequently, the sequences of *groEL* (874 bp), 16S rRNA (1261 bp), *gltA* (594 bp), and *msp4* (656 bp) of *A. capra* were obtained and submitted to GenBank (Accession Nos. MT804297, MT799937, MT804296, and MT804298, respectively). The entire 16S rRNA sequence of the *Anaplasma* sp. from a goat was
100% homologous to sequences observed in a human (GenBank Accession No. KM206273) and H. longicornis (GenBank Accession No. KY242456). The phylogenetic tree, based on 16S rRNA sequences, showed that the isolated Anaplasma sp. nov is in the same clade with A. capra from R. microplus (MH762071), H. longicornis (KY242456), H. sapiens (KM206273), goat (MG869483), sheep (MF066917), cattle (MF000918), and Korean water deer (KM206275, KM206277) (Figures 3 and 4(A,B)). Phylogenetic tree analysis based on the sequences of gltA, msp4, and groEL genes indicated that the Anaplasma sp. sequence detected in the present study is in the same clade with A. capra sequences previously reported (Figures 3 and 4(B)). However, the sequence from the French red deer and swamp deer appeared to have a different genotype, which was in the same cluster with “A. capra,” but on a different branch (Figures 3 and 4(B)). Moreover, phylogenetic trees based on the four gene sequences showed that A. capra is in the same cluster with A. marginale, A. centrale, and A. ovis, suggesting that A. capra and the three Anaplasma spp. may have some similar characteristics.

The erythrocytes infected with A. capra were examined using electron microscopy. SEM images demonstrated the presence of one or more A. capra cells (0.2–0.4 µm in diameter) on the outer surface of the erythrocytes (Figure 5(A2–A4)). Additionally, invasion of the erythrocytes was also observed (Figure 5(A2)). TEM images showed one or more typical small round to oval morulae (0.2 × 0.4 µm) on the membrane and in multiple erythrocytes (Figure 5(B1–B4, C1–C4)). The morulae in the cytoplasm of the erythrocytes were about 10 times (0.8 × 1 µm) the size of those on the outside cells (Figure 5(C1–C4)). The morulae density was not uniform, and electron-dense particles (Lysosomes) were observed in less-dense areas of the pathogen (Figure 5(C2–C4)). When separated from erythrocytes, the morulae consisted of a membrane-bound vacuole containing dense granular bacterial subunits with a lower pathogen density than intracellular morulae, and the lysosomes were absent (Figure 5(D1–D4)).

Wright–Giemsa-stained A. capra-positive erythrocytes smear showed numerous small morulae in the cytoplast (Figure 6(A1–A2)). In the CISH assay, microscopic observation demonstrated reddish-brown (DIG) specific probe signals on the surface and inside of A. capra-positive erythrocytes (Figure 6(B1)). The negative controls showed erythrocytes with non-specific probe signals (Figure 6(B2)).

Immunocytochemistry-positive cells were observed in erythrocytes, and appeared as intracytoplasmic aggregates, known as morulae (Figure 6(C1–C2)). No morulae were observed in the negative controls. In addition, immunocytochemistry-positive cells were occasionally noted in neutrophilic granulocytes, as the whole blood cells were also Anaplasma phagocytophilum-positive.

IFA positive fluorescence was obtained with A. capra antiserum, confirming that the cells contained A. capra parasites (Figure 6(D2)). In contrast, negative-control erythrocytes showed no fluorescence (Figure 6(D1)). Thus, owing to its unique morphological characteristics and parasitic site, we propose to
Anaplasma capra sp. nov was first identified from seven goats based on the 16S rRNA gene in Southwest China. Sequences from the goats formed two Anaplasma spp. clusters that clearly distinguished them from the clusters of A. marginale, A. centrale, and A. ovis, suggesting that this pathogen could be a potential new Anaplasma sp. [2]. Similarly, Liu et al. [17] (2012) performed molecular analysis of the pathogen based on 16S rRNA gene in goats from Zhejiang, China. A comparable genotype of this Anaplasma sp. was detected in humans with tick-bite history since 2015 based on groEL, 16S rRNA, gltA, msp2, and msp4 genes [1], and was provisionally nominated as “A. capra,” belonging to the Genus Anaplasma. Likewise, we found that A. capra sp. nov exhibited significant differences in groEL, 16S rRNA, gltA, and msp4 genes, when compared with other Anaplasma spp. [5]. Many studies have used this molecular method to identify and characterize A. capra sp. nov in different hosts [6,7,24–26].
Figure 5. Photomicrographs of *A. capra* sp. nov and infected goat erythrocytes. A1–A4, SEM photomicrographs of erythrocytes and *A. capra* sp. nov. Normoerythrocytes (A1) and infected erythrocytes. Arrows indicate *A. capra* sp. nov (A2–A4). A2 shows invading pathogen (red arrowhead). B1–B4 and C1–C4, TEM photomicrographs of erythrocytes and *A. capra* sp. nov. Uranyl acetate and Reynold’s lead citrate stain. Morulae are observed beside and within multiple erythrocytes (arrows). B2–B4 and C2–C4 show higher magnification of the morulae. Electron-dense particles (Lysosomes, arrows) are observed in less-dense areas (C2–C4, arrows). D1–D4, TEM photomicrographs of the separated *A. capra* sp. nov morulae. D1 (arrows) shows lower magnification of the morulae and D2–D4 present higher magnification of the morulae. No Lysosomes are observed in the morulae.

Figure 6. Wright–Giemsa, CISH, immunocytochemistry, and IFA analyses of uninfected and infected goat erythrocytes. A1–A2, Wright–Giemsa-stained erythrocytes. B1–B2, CISH assay of the erythrocyte smear. A1–D1 are negative controls. The probe was labeled with DIG. C1–C2 and D1–D2, immunocytochemistry and IFA of erythrocytes incubated with positive goat serum. Black and white arrows denote *A. capra* sp. nov-positive erythrocytes. Red arrows show *A. phagocytophilum*-positive neutrophilic granulocytes.
Anaplasma capra sp. nov appears to exhibit at least two different genotypes, both are likely zoonotic [4, 7]. The sequences of gltA and groEL genes examined in the present study formed two clusters in the phylogenetic tree. In a previous study, sequence analysis based on gltA gene demonstrated a novel A. capra sp. nov genotype in sheep, which was distinct from the isolates identified from patients in northeastern China [27]. Furthermore, our previous study also identified two genotypes based on gltA gene of A. capra sp. nov in sheep and goats from Henan, China [5]. Additionally, it has been reported that sequences based on the 16S rRNA gene from goat strains and the gltA gene from sheep strains formed two distinct A. capra sp. nov clusters [2,28]. Hence, our future research will involve genotyping A. capra sp. nov. As both the genotypes of A. capra sp. nov have been detected in ruminants and hard ticks, they pose a potential health threat to humans. The host range of the zoonotic genotypes needs further study, especially in areas infested with hard ticks.

Anaplasma spp. that have been reported to infect erythrocytes include Anaplasma marginale, A. centrale, and A. ovis [29–31]. A. marginale and A. centrale were first identified in 1910 and 1911, respectively, by Theiler, who observed small morulae of A. marginale that was previously mistakenly as Babesia bigemina at the periphery of stained cattle erythrocytes [18,32]. Anaplasma centrale forms smaller and more central morulae and is closely associated with A. marginale within infected erythrocytes [30], while A. ovis was first detected in the central area of sheep erythrocytes in 1912 [31]. However, there are still no relevant reports on the specific site location of A. capra sp. nov. Similar to other Anaplasma spp., the target cells of this pathogen may be one type of blood cell. Li et al. presumed that A. capra sp. nov most probably infect erythrocytes or endothelial cells in mammals; however, this assumption has not yet been proved [1]. Interestingly, in the present study, A. capra sp. nov was detected inside or at the periphery of erythrocytes that were A. capra sp. nov PCR-positive and A. marginale, A. centrale, and A. ovis PCR-negative, and could infect human erythrocytes. The morphological characteristics of A. capra sp. nov determined in the present study are consistent with those previously reported [1], and different from the other intraerythrocytic Anaplasma spp. [20,23,33–35]. These findings significantly contribute to the research on this novel zoonotic Anaplasma sp.

Erythrocytes infected with intraerythrocytic Anaplasma spp. are destroyed by macrophages, resulting in mild to severe hemolytic anemia [18]. Sheep and goats infected with Anaplasma spp. develop a mild to severe disease, with clinical symptoms such as fever, pale mucous membranes, weight loss, icterus, anorexia, depression, lower milk production, coughing, dyspnea, gastrointestinal signs, abortion, and death [36,37]. Humans infected with A. capra sp. nov exhibit fever, headache, malaise, dizziness, chills, nausea, vomiting, diarrhea, and laboratory abnormalities, e.g. high hepatic aminotransferase concentrations, leucopenia, and thrombocytopenia [1]. The pathogen is carried and may be transmitted to their zoonotic and domestic hosts by many species of hard ticks that are widely distributed [9–12,38–41]. Thus, A. capra sp. nov is a substantial threat to humans and domestic animals, and precautions should be taken to prevent anaplasmosis caused by this novel tick-borne pathogen.

Conclusion

To our knowledge, this was the first study to detect a novel zoonotic tick-borne Anaplasma sp., A. capra, in host erythrocytes. The molecular characteristics, morphological features, and parasitic sites of A. capra were noted to differ from those of other Anaplasma spp. Hence, we propose revising the Genus Anaplasma and formally naming “A. capra” as A. capra sp. nov.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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