Insight into the genetic diversity of *Anaplasma marginale* in cattle from ten provinces of China

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**Abstract**

**Background:** *Anaplasma marginale* is an important tick-transmitted rickettsial pathogen of cattle, with worldwide distribution and an important economic impact. The genetic diversity of *A. marginale* strains has been extensively characterized in different geographical regions throughout the world, while information is limited on studies in China. This study was carried out to determine the prevalence and genetic diversity of *A. marginale* strains in cattle from ten provinces of China.

**Methods:** A total of 557 blood samples from cattle were collected and screened for the occurrence of *A. marginale* by PCR based on the *msp4* gene. The partial *msp1a* gene containing tandem repeat sequences was further amplified from *msp4* positive samples. The Msp1a amino acid repeats were identified, and genetic variation of *A. marginale* strains was characterized based on the variation in the repeated portion of Msp1a.

**Results:** Our results showed that 31.6% of 557 cattle were positive for *A. marginale*. The infection rates of *A. marginale* varied considerably from 0 to 96.9% in different sampling regions. Sequence analysis revealed that two *msp4* sequence variants of *A. marginale* exist in cattle. One hundred and three *msp1a* sequences were obtained and permitted to identify 42 Msp1a tandem repeats, 21 of which were not previously published for *A. marginale*. Moreover, 61 *A. marginale* genotypes were identified based on the structure of Msp1a tandem repeats.

**Conclusions:** *Anaplasma marginale* is widely distributed in China and a high prevalence of infection was observed in cattle. The geographical strains of *A. marginale* were molecularly characterized based on the structure of Msp1a tandem repeats. Forty-two Msp1a tandem repeats and 61 genotypes of *A. marginale* were identified. This study, for the first time, revealed the genetic diversity of *A. marginale* strains in cattle in China.

**Keywords:** *Anaplasma marginale*, msp4 gene, Msp1a tandem repeats, Genotypes, Cattle, China
vary in genotype, virulence, antigenic characteristics and infectivity for ticks [6]. Characterization of the genetic diversity of A. marginale strains has been performed based on the variability of tandem repeat amino acid sequences located in the N-terminal region of the major surface protein (Msp) 1a, and numerous geographical Msp1a tandem repeats and genotypes were identified [10]. In China, A. marginale has been recognized for over 30 years, and Rhipicephalus microplus is considered to be the most important tick vector with a nationwide distribution [11, 12]. Despite the importance of bovine anaplasmosis, limited information is available for A. marginale in China. Previously, the occurrence of A. marginale was reported in several provinces, and only one Msp1a tandem repeat (GenBank: DQ811774) was identified in A. marginale strain HB-A8 from cattle [11–15]. The objective of this study was to determine the prevalence and genetic diversity of A. marginale strains in cattle from different geographical areas of China.

Methods
Study areas, sample collection and DNA isolation
This study was conducted between 2011 and 2015 in rural areas of 22 counties from ten provinces of China, including Inner Mongolia and Liaoning (north-east China); Hunan, Guangdong, Guangxi and Hainan (south-central China); Chongqing, Sichuan, Guizhou and Yunnan (south-west China). The sample sites are listed in Table 1. Animals for this study were randomly selected in two to three herds for each county. A total of 557 jugular blood samples were collected in vacutainer EDTA tubes from adult cattle. Genomic DNA was prepared from 300 μl blood samples using the Gentra Puregene Blood Kit (Qiagen, Beijing, China) following the protocols recommended by the producer. DNA was resuspended in the elution buffer provided in the commercial kit and stored at -20 °C until use.

PCR reactions
The extracted DNA was used for the amplification of msp4 gene of A. marginale by nested PCR [16, 17]. Briefly, the primers MSP43 (5′-GGG AGC TCC TAT GAA TTA CAG AGA ATT GTT TAC-3′) and MSP45 (5′-CCG GAT CCT TAG CTA AAC AGA ATC TTG C-3′) were used for the first round of PCR amplification, while AmargMSP4Fw (5′-CTG AAG GGG GAG TAA TGG G-3′) and AmargMSP4Rev (5′-GGT TAT AGC CCC TCG CAG AGA ATC TTG C-3′) were used in a nested-PCR reaction, which generated a fragment of 344 bp. The DNA extracted from cattle infected with A. marginale (isolate Lushi, GenBank: AJ633048) and sterile water was used as the positive and negative control, respectively. The partial msp1a gene containing the tandem repeats of A. marginale was further amplified from msp4-positive samples by PCR as reported previously [18] with some modifications. The outer primers 1733F (5′-TGT GCT TAT GGC AGA CAT TTC C-3′) and 3134R (5′-TCA CGG TCA AAA CCT TTG CTG ACC-3′) were used in the first reaction as described by Lew et al. [18]. An inner forward primer AM-F2 was designed in highly conserved region of msp1a sequences available in GenBank using OligoAnalyzer 3.1 (Integrated DNA Technologies, 2012, Iowa, USA). The inner primers AM-F2 (5′-CTT AGA GGT GTT GTG TAC C-3′) and 2957R (5′-CAA CCT TTG TGG TAC C-3′) were used in the second reaction in this study and 2957R (5′-AAA CCT TGT AGC CCC AAC TTA C-3′) were used in the second reaction [18]. The reactions were performed in an automatic thermocycler (Bio-Rad, Hercules, USA) with a final volume of 25 μl containing 2.0 μl template DNA. Thermal cycling comprised 4 min of an initial denaturation at 94 °C, 35 cycles of 94 °C for 30 s, annealing for 30 s (55 °C for 1733F/3134R, 60 °C for MSP43/MSP45, AmargMSP4Fw/AmargMSP4Rev and AM-F2/2957R)
and 72 °C for 30–90 s (depending on the target fragments), and a final extension at 72 °C for 10 min. Amplified products were analyzed by 1.0% agarose gel electrophoresis.

**Sequences and statistical analysis**

The purified PCR amplicons of *msp4* and *msp1a* genes of *A. marginale* were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Two recombinants were selected randomly and sequenced (GenScript, Nanjing, China). Sequence analysis was performed using the BLASTn search and the ClustalW software (DNASTar, Madison, WI, USA). The *A. marginale msp1a* sequences were trimmed and translated to amino acids using CLC Genomics Workbench 7.5.1 (Qiagen, Aarhus, Denmark). The tandem repeats of *A. marginale* Msp1a amino acid sequences were identified and aligned by using the ClustalW software. Statistical analysis was conducted using a Chi-square test in PASW statistics 18.0 (SPSS, Chicago, IL, USA). P-values of 0.05 or less were considered statistically significant.

**Nucleotide sequence accession numbers**

The sequences obtained in this study were submitted to the GenBank database and provided accession numbers as follows: MF326686 and MF326687 for *msp4* and MF326688–MF326790 for *msp1a*.

**Results**

*Anaplasma marginale* DNA was detected in 176 of 557 cattle, with an overall infection rate of 31.6% (Table 1). The infection rates of *A. marginale* varied considerably from 0 to 96.9% in different sampling regions. The infection was detected in 17 of 22 counties, representing all ten provinces included in this study. The infection rate of *A. marginale* in the south-west (67/183, 36.6%) was almost comparable with that in the south-central region (102/300, 34.0%) (χ² = 0.163, df = 1, P > 0.05), but was significantly higher than in the north-east (77/240, 31.9%) (χ² = 11.621, df = 1, P < 0.001).

Sequence analysis of *msp4* gene confirmed the infections of *A. marginale* in cattle, and two *msp4* sequence variants with 99.7% similarity were obtained in this study. The *msp4* sequence variant 20-14c (GenBank MF326686) was identical to the *A. marginale* strains Tamaulipas, Kanchanaburi66 and 11-MSP43 (GenBank: MF326686) was identical to the *msp4* study. The variants with 99.7% similarity were obtained in this study.

On the basis of the *msp4* PCR results, *A. marginale*-positive samples were subjected for further analysis. One hundred and three *msp1a* sequences (GenBank: MF326688–MF326790) were obtained. Sequence analyses revealed that 97.1% (100/103) of *A. marginale* isolates contained the Msp1a tandem repeats, and 42 different types of Msp1a tandem repeats with 28 to 29 amino acids among Chinese *A. marginale* strains were identified (Fig. 1). Aside from Msp1a tandem repeats (M, F, r, Ph9, Is1; 73, 13, 27, MGl10, 154, 103; Me1, 14, 72; 80, C, 3, 17, 10, LJ1, 22–2, 37, 4 and Ph2) with known name reported in previous studies [21], 21 new tandem repeats (designated as Ch1–21; Fig. 1) are described for the first time in this study.

The genetic diversity of *A. marginale* strains was analyzed based on the Msp1a tandem repeats structure. A total of 103 *A. marginale* isolates were classified into 61 genotypes with a maximum repeat number of five (Table 2). Interestingly, three isolates (AM5-2a, AM5-2b and AM17-2b; GenBank: MF326718, MF326719 and MF326770) had no amino acid repeats (Table 2). The remaining 100 isolates contained one to five Msp1a tandem repeats. As shown in Table 2, five Msp1a tandem repeats were identified in five *A. marginale* isolates; four repeats in 23 isolates; three repeats in 26 isolates; two repeats in 32 isolates and a single repeat in 14 isolates (Table 2). Most of these Msp1a tandem repeats (Ch1, F, M, Ph9, etc.) were shared between different *A. marginale* isolates and genotypes, while some of them (Ch4, Ch5, Ch7, etc.) were unique and had a low frequency among these isolates (Table 2). In addition, 21 animals positive for *A. marginale* identified in this study were infected by more than one genotype.

**Discussion**

Bovine anaplasmosis caused by *A. marginale* is widely distributed in tropical and subtropical areas throughout the world [22]. In China, *A. marginale* was first isolated from cattle as early as 1987 in Lushi County, Henan Province [11]. Since then, *A. marginale* has been detected in *Hyalomma asiaticum* ticks and cows from five farms in northwestern China [13]. A molecular survey of *Anaplasma* spp. has previously been conducted in domestic ruminants from 12 provinces of China, and *A. marginale* infection in cattle was identified by *gltA* sequencing [14]. In addition, this agent has also been found in cattle from Chongqing, southwestern China [15]. Those reports provided molecular evidence of *A. marginale* by genus-specific PCR and sequencing in domestic ruminants in China. However, information of epidemiology and molecular characterization of Chinese strains is limited. In the present study, a molecular survey of *A. marginale* was conducted by species-specific PCR in cattle, and 31.6% of 557 sampled animals were naturally infected with this organism. Since animals infected by *A. marginale* can develop a persistent infection that may facilitate the maintenance and further spread of infection [23], a high prevalence of *A. marginale* was
relatively common in the vertebrate hosts. In this study, a significant difference in infection rates of *A. marginale* was observed between the South and the North area of China, and this may be mainly associated with the tick vectors. The geographical distribution of different tick species in China vary from South to North due to the diverse ecological environments, climate variability and hosts [24], affecting consequently the presence of tick-borne diseases. *Anaplasma marginale* was identified in all ten sampled provinces, indicating that this agent was widely distributed and may pose a serious threat to the cattle industry in China, which should arise extensive attention.

The members in the genus *Anaplasma* have diverse surface-exposed proteins [6]. There are six major surface proteins (MSPs) that have been well characterized in *A. marginale*, and were considered to be involved in the interactions of pathogen with both ticks and hosts [22, 25]. These major surface protein genes may evolve more obviously because of the selective pressure exerted by the host immune system [26]. The genetic variability of *A. marginale* was frequently characterized on the basis of the *msp4* and *msp1a* genes [27]. However, the *msp4* gene is highly conserved and stable among widely divergent strains of *A. marginale* [28]. In this study, the *msp4* sequences of *A. marginale* isolates identified in cattle from different geographical regions shared high sequence identity (99.7 to 100%), and have previously been reported in cattle from other countries [19, 20].

**Fig. 1** Alignment of Msp1a amino acid repeat sequences of *A. marginale* detected from Chinese cattle. The 42 repeat types were aligned using the ClustalW method in the MegAlign software. The Msp1a tandem repeats (M, F, t, Ph9, Is1; 73, 13, 27, MGl10, 103, Me1, 14, 72; 80, C, 3, 17, 10, LJ1; 22–2, 37, 4 and Ph2) identified herein have been reported in previous studies, and 21 new Msp1a tandem repeats were named as Ch1–21. The one letter code was used to reveal the different amino acid sequences of Msp1a repeats. The variable amino acids are highlighted on a black background and gaps indicate deletions/inversions.
### Table 2 Organization of Msp1a tandem repeats in *A. marginale* strains identified in cattle

| Strains | GenBank ID | Structure of Msp1a tandem repeats |
|---------|------------|----------------------------------|
| AM1-10a | MF326688   | Ch1 M                            |
| AM1-10b | MF326689   | Ch1 F M M                        |
| AM1-102a| MF326690   | Ch1                              |
| AM1-102b| MF326691   | Ch1                              |
| AM3-10a | MF326692   | t M Ch2                          |
| AM3-21a | MF326693   | Ph27 Is1; 73; Is1; 73; Is1; 73    |
| AM3-21b | MF326694   | Ph27 Is1; 73; Is1; 73; Is1; 73    |
| AM3-27a | MF326695   | 13 27 27 27                      |
| AM3-27c | MF326696   | 13 27 27                        |
| AM4-1a  | MF326697   | Ch1                              |
| AM4-1b  | MF326698   | Ch1                              |
| AM4-2a  | MF326699   | Ch3 Ch2 Ch2                     |
| AM4-4a  | MF326700   | Ch1                              |
| AM4-4c  | MF326701   | Ch1 M F M                        |
| AM4-6a  | MF326702   | MGII10 154                      |
| AM4-7a  | MF326703   | 2Is1; 73 Is1; 73                 |
| AM4-8a  | MF326704   | 2Is1; 73 Is1; 73                 |
| AM4-9a  | MF326705   | 2Is1; 73 Is1; 73                 |
| AM4-10b | MF326706   | 2Is1; 73 Is1; 73                 |
| AM4-12a | MF326707   | Ch4 Ch5                          |
| AM4-12b | MF326708   | Ch4 Ch5                          |
| AM4-15a | MF326709   | Ch6 Ch2 Ch2 Ch2 Ch2             |
| AM4-15b | MF326710   | M M 103; Me1                     |
| AM4-17b | MF326711   | Ph9 Is1; 73                      |
| AM4-18a | MF326712   | Ch7 14                           |
| AM4-18b | MF326713   | Ch7 14                           |
| AM4-21a | MF326714   | 72; 80 Ch8 Ch8 Ch8 Ch8 Ch8       |
| AM4-22b | MF326715   | Ch7 14                           |
| AM4-23b | MF326716   | 27 Is1; 73 Is1; 73               |
| AM4-24a | MF326717   | Ch9 Ch3 Ch3 Ch3                  |
| AM5-2a  | MF326718   |                                 |
| AM5-2b  | MF326719   |                                 |
| AM5-4a  | MF326720   | Ch3 Ch2 Ch2                      |
| AM5-4b  | MF326721   | Ch1 M                            |
| AM5-6a  | MF326722   | 13                               |
| AM5-6b  | MF326723   | Ch3 Ch2 Ch2 Ch2 Ch2             |
| AM5-8a  | MF326724   | F M M                            |
| AM5-8b  | MF326725   | Ch1                               |
| AM5-9b  | MF326726   | Ch1 M F M                        |
| AM5-11b | MF326727   | Ph9 Is1; 73 Ch2                  |
| AM5-11c | MF326728   | Ph9 Is1; 73 Is1; 73 Is1; 73      |
| AM5-13c | MF326729   | 27 Is1; 73                       |

(Continued)

| Strains | GenBank ID | Structure of Msp1a tandem repeats |
|---------|------------|----------------------------------|
| AM5-15a | MF326730   | 27 Is1; 73 Is1; 73               |
| AM5-15c | MF326731   | 27 Is1; 73                       |
| AM5-16b | MF326732   | F M C                            |
| AM5-19a | MF326733   | Ch1 M F M                        |
| AM5-19b | MF326734   | Ch1 M F M                        |
| AM5-22a | MF326735   | 13 14 M                          |
| AM5-22c | MF326736   | Ch1 M F M                        |
| AM6-7a  | MF326737   | Ch10 Is1; 73 Is1; 73             |
| AM6-7b  | MF326738   | Ch10 Is1; 73 Is1; 73             |
| AM7-8b  | MF326739   | F M M                            |
| AM8-5c  | MF326740   | 27 Is1; 73 24 Is1; 73           |
| AM9-3b  | MF326741   | 3                                |
| AM9-3c  | MF326742   | 103; Me1 3 3                     |
| AM9-5a  | MF326743   | 13 17 Ch2                        |
| AM9-14a | MF326744   | F 10                             |
| AM9-14b | MF326745   | F 10 MG110                      |
| AM9-21a | MF326746   | L1 22–2 27 14                   |
| AM9-24a | MF326747   | M F                              |
| AM9-24b | MF326748   | Ph9 Is1; 73 Is1; 73 Is1; 73      |
| AM9-26b | MF326749   | 13                               |
| AM9-26c | MF326750   | 37 154 27                       |
| AM15-3b | MF326751   | Ph9 Is1; 73                      |
| AM15-5a | MF326752   | 27 Is1; 73 Is1; 73 Is1; 73       |
| AM15-5b | MF326753   | 27 Is1; 73 Is1; 73 Is1; 73       |
| AM15-18a| MF326754   | Ch12 Is1; 73                     |
| AM15-18b| MF326755   | Ch12 Ch13 Is1; 73                |
| AM15-23b| MF326756   | 3 Ch2                            |
| AM15-30a| MF326757   | M M M M                         |
| AM15-30b| MF326758   | 13 4                             |
| AM16-1b | MF326759   | Ch14 Ch2                         |
| AM16-2b | MF326760   | 13 14                            |
| AM16-2c | MF326761   | 13 14                            |
| AM16-5c | MF326762   | Ph9                              |
| AM16-9b | MF326763   | Ch15 Is1; 73                     |
| AM16-12b| MF326764   | Ch10 Is1; 73 Is1; 73             |
| AM16-14a| MF326765   | 13 14                            |
| AM16-14b| MF326766   | 13 14                            |
| AM16-21b| MF326767   | 13 14                            |
| AM16-25a| MF326768   | Ch16 Ch17 Ch17 Is1; 73 Ch2       |
| AM16-25b| MF326769   | Ch18 Ch19 Is1; 73 Ch2            |
| AM17-2b | MF326770   |                                 |
| AM18-3b | MF326771   | 3 Is1; 73 Is1; 73                |
permitted identification of 42 Msp1a tandem repeats, 50% of which were identical to those previously published for *A. marginale* strains. The Msp1a tandem repeats were not always clustered together corresponding to the geographical locations; some repeats have been identified in the *A. marginale* isolates from various regions and appeared to be distributed nationwide (Table 2). These findings suggest that there is no significant association between specific Msp1a repeats and geographical regions, and this may be attributed to movement of vectors and vertebrate hosts.

*Anaplasma marginale* geographical strains differ in the copy number and amino acid repeat sequences in Msp1a [29]. In our study, 61 *A. marginale* genotypes were identified based on the variation in the repeated portion of Msp1a, showcasing the broad genetic diversity of *A. marginale* in cattle in China. Previous reports have demonstrated that the Msp1a repeats contain functional domains that are involved in adhesion to tick cells and bovine erythrocytes [30]. They also contain B cell and neutralization epitopes that are critical for immune protection in animals [30], suggesting that Msp1a repeats play an important role in the invasion, transmission and survival of *A. marginale*. Generally, *A. marginale* strains contain at least one Msp1a tandem repeat (maximum number of 10) [6]; however, the repeat sequence was not found in three isolates from Guangdong and Guangxi Province in south-central China.

It has been demonstrated that the animals and ticks naturally infected with one genotype of *A. marginale* preclude infection with additional genotypes, indicating that different genotypes could not coexist in the same animals and ecosystems [31, 32]. This infection exclusion mechanism has also been revealed for *Rickettsia* species [33]. However, *A. marginale* strain superinfection with different Msp1a genotypes has been reported subsequently and proven to be associated with high levels of infection prevalence [34–36]. In the present study, 21 animals positive for *A. marginale* were infected by multiple genotypes. This finding was consistent with the previous report [37], in which described distinct *A. marginale* strains circulated in the same animals and herd. A similar phenomenon was also observed for *A. marginale* subsp. *centrale* [38]. The coexistence of divergent *A. marginale* strains may serve as a potential source of variation.

In summary, our results revealed the prevalence and genetic diversity of *A. marginale* strains using Msp1a tandem repeats in ten provinces. As one of the most important tick-borne diseases, bovine anaplasmosis caused by *A. marginale* should no longer be neglected in endemic areas of China.

### Conclusions

In the present study, 31.6% of 557 cattle from 22 counties of ten provinces were positive for *A. marginale*. The *A. marginale* strains were molecularly characterized based on the structure of Msp1a amino acid repeats. A total of 103 isolates were classified to 61 genotypes, and 42 Msp1a tandem repeats were identified, 21 of which have not previously been described. The present study, for the first time, revealed the genetic diversity of *A. marginale* strains using Msp1a repeat sequences in cattle in China.

### Abbreviations

EDTA: ethylene diamine tetraacetic acid; Msp: major surface protein; UV: ultraviolet

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### Availability of data and materials

The sequences obtained in this study were submitted to the GenBank database and provided accession numbers as follows: MF326686 and MF326687 for msp4 and MF326688–MF326790 for msp1a.

### Authors’ contributions

HY and JY designed and coordinated this study. JY and RH drafted and revised the manuscript. JY, ZL, QN and GG collected the samples included in their respective provinces.
this study. RH, JY, QN, GL and JL conducted the experiments and data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Collection of cattle samples was approved by the owner, and animals were handled in accordance with the Animal Ethics Procedures and Guidelines. The study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute (Approval No. LYRIM2021-018).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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