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Molecular detection and genotyping of *Anaplasma* spp. and *Theileria* spp. infections in sheep and cattle from the northeast region of China

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Abstract. Anaplasmosis and theileriosis are significant tick-borne diseases threatening the livestock industry worldwide. In the present study, we screened 127 cattle and 115 sheep blood DNA samples from northeastern China for *Theileria* and *Anaplasma* pathogens by polymerase chain reaction (PCR) using species-specific primers. The result showed that only *Theileria orientalis* and *Anaplasma ovis* were detected, with a prevalence of 2.9% for *T. orientalis* in cattle and 57.4% for *A. ovis* in sheep. Fragments of *Anaplasma ovis* major surface protein 4 (AoMSP4) and *Theileria orientalis* major piroplasm surface protein (ToMPSP) genes were sequenced for phylogenetic analysis. Sequence analysis showed that the AoMSP4 gene was conserved, with 100% sequence identity value among sheep samples. However, the ToMPSP gene was relatively diverse, with sequence identity ranging from 87.6%–99l.0% among cattle samples. Phylogenetic analysis showed that the ToMPSP gene sequences isolated from 4 cattle samples were classified into type 1, type 2 and type 7, while the AoMSP4 gene sequences obtained from 66 sheep were classified into genotype I, according to the neighbour-joining distance method. This study provides important data for understanding the epidemiology of tick-borne diseases and genetic diversity of these pathogens in the northeast region of China.

INTRODUCTION

Tick-borne diseases pose a huge threat to the livestock industry in many countries of the world (Jongejan & Uilenberg, 2004; Jensenius *et al.*, 2006). Among all the tick-borne diseases, babesiosis, theileriosis and anaplasmosis are the most widely distributed, and economically important diseases affecting cattle and sheep (Uilenberg, 1995; de Castro, 1997; Dantas-Torres *et al.*, 2012).

Theileriosis can be classified into malign and benign forms based on the pathogenicity of different *Diphtheria* SPSS. (Onuma *et al.*, 1998). The malign parasites, *Theileria annulata* and *Theileria parva*, are considered the most pathogenic species to cattle, but their transmission is limited by the distribution of their tick vectors; *Hyalomma* spp. and *Rhipicephalus appendiculatus* (Mukhebi *et al.*, 1992; Bilgic *et al.*, 2010; Weir *et al.*, 2011). *Theileria orientalis* is thought to be a benign, less pathogenic parasite, which is widely distributed in tropical and subtropical areas (Onuma *et al.*, 1998; Altangerel *et al.*, 2011). However, infected animals may present severe clinical symptoms in some cases (Aparna *et al.*, 2011).

Anaplasmosis is an infectious disease caused by members of the intraerythrocytic bacteria of the genus *Anaplasma*. The major species affecting livestock are *Anaplasma marginale*, *A. bovis*, *A. phagocytophilum*, and *A. ovis* (Liu *et al.*, 2012). *A. marginale* and *A. ovis* are the main causative agents of ovine anaplasmosis (Fuente *et al.*, 2007; Torina *et al.*, 2012). *A. ovis* infection is usually asymptomatic. The clinical signs usually develop in the event of immunosuppression in sheep, characterized by severe anemia, fever, weight loss, abortion, pallor of mucous membrane and jaundice (Kocan *et al.*, 2003).

The *T. orientalis* major piroplasm surface protein (ToMPSP) gene has been widely used as an epidemiological molecular marker for genotyping of *T. orientalis*. Eleven genotypes (Type 1-8 and TypeN1-N3) of *T. orientalis* have been reported based on the phylogenetic analysis (Ota *et al.*, 2009; Altangerel *et al.*, 2011). The genetic diversity of *A. ovis* strains has been well characterized based on *A. ovis* major surface protein 4 (AoMSP4) gene. Seven genotypes (Type I-VII) have been reported based on phylogeny with previously reported sequences (Liu *et al.*, 2012; de la Fuente *et al.*, 2007).

Cattle and sheep are the major economically important livestock in the northeast region of China. Theileriosis and anaplasmosis are the most prevalent tick-borne diseases which have been reported to be widely distributed in China (Liu *et al.*, 2012; Cao *et al.*, 2013; Qiu *et al.*, 2016; Qin *et al.*, 2016). Previous studies have identified three bovine *Theileria* species in China: *T. annulata*, *T. orientali*s and *T. sinensis* (Liu *et al.*, 2010; Liu *et al.*, 2015), while *A. marginale*, *A. ovis*, and *A. phagocytophilum* were reported in small ruminants (Qiu *et al.*, 2016). However, there is limited information regarding the presence and genetic diversity of these causative agents in the northeast region of China. Therefore, the aim of the present study is to investigate the presence and genotypes of these tick-borne pathogens in cattle and sheep in order to have a better understanding on their distribution.

MATERIALS AND METHODS

Sample collection and DNA isolation

The cross-sectional study was conducted in June 2016. Blood samples were collected from cattle (n=127) and sheep (n=115) from two provinces in the northeast region of China, including Inner Mongolia and Heilongjiang. For cattle, 90 samples were collected from Inner Mongolia and 37 were collected from Heilongjiang. For sheep, all samples were collected from Heilongjiang. All samples were collected from randomly selected male and female herds. The animals were restrained in crush and 5 ml of blood sample was collected from the jugular or caudal vein of each animal and immediately transferred into 10 ml vacuum-blood collection tube with EDTA. The samples were kept at 4°C in a cool box and transported with ice pack to the laboratory. DNA was extracted from 200 µl of each blood sample using Qiagen blood DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions and stored at 30°C.

PCR assays for detection of *Theileria* spp. and *Anaplasma* spp.

Cattle infected by *T. annulata*, *T. orientalis* and *A. phagocytophilum* and small ruminants infected by *T. orientalis*, *A. phagocytophilum* and *A. ovis* were previously reported in China (Liu *et al.*, 2012; Cao *et al.*, 2013; Qiu *et al.*, 2016; Qin *et al.*, 2016). Therefore, in the study, each cattle sample was screened using species-specific PCR assays for detection of *T. annulata*, *T. orientalis*, *A. phagocytophilum* and each sheep sample was screened by species-specific PCR assays for detection of pathogens including *T. orientalis*, *A. phagocytophilum* and *A. ovis* as previously described (D'Oliveira *et al.*, 1995; Ota *et al.*, 2009; Walls *et al.*, 2000; Torina *et al.*, 2012). The target genes were *T. annulata* merozoite surface antigen (TaMSA), *T. orientalis* major piroplasm surface protein (ToMPSP), *A. phagocytophilum* epank1 (Ap*epank1*) encoding 4 of the 11 ankyrin repeats, a region

comprising 444 nucleotides and *A. ovis* major surface protein 4 (AoMSP4) (Table 1). Single-step PCR assays were employed to detect all the surveyed pathogens as described in previous studies. The PCR products were separated by gel electrophoresis on 1.5% agarose in 1× TAE buffer and visualized using ethidium bromide using a UV transilluminator. The positive control were positive samples for previous studies (Zhou *et al.*, 2016), while double distilled water (DDW) was used as negative control.

Cloning and sequencing

T. orientalis (n=4) and *A. ovis* (n=8) positive samples were randomly selected for DNA cloning and sequencing. PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Germany), inserted into pGEM-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* DH5α-competent cells. Three positive clones were selected for sequencing using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and the DNA sequences were determined using ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA). The nucleotide sequences were analyzed using Bioedit version 7.2.5 (Tom Hall Ibis Biosciences, USA) and their identities and similarities were determined by GenBank BLASTn analysis. The percent identities between nucleotide were calculated by Pairwise distances using the MEGA version 6.0 program (Tamura *et al.*, 2013).

Phylogenetic tree analysis

The genetic relatedness between *T. orientalis* and *A. ovis* isolates in northeastern China and those from other regions of the world was established by phylogenetic analyses using the MEGA version 6.0 program. The neighbor-joining distance method was used to construct phylogenetic trees for *Theirelia* spp. and *Anaplasma* spp.. Bootstrap analysis with 1000 replications was used to estimate the confidence of branching patterns of the trees.

Nucleotide sequence accession numbers

The GenBank accession numbers of sequences obtained in this study were as follows: KY495506, KY495507, KY495508 and KY495509 for the *T. orientalis* MPSP gene and KY511046 for the *A. ovis* MSP4 gene.

RESULTS

Detection of *Theileria* spp. and *Anaplasma* spp. in cattle and sheep blood samples

In the study, *T. annulata* merozoite surface antigen (TaMSA) and *A. phagocytophilum* epank1 (Ap*epank1*) genes were not detected, only *T. orientalis* major piroplasm surface protein (ToMPSP) and *A. ovis* major surface protein 4 (AoMSP4) genes were amplified from the cattle or sheep blood samples. The prevalence of *T. orientalis* and *A. ovis* infections in cattle and sheep is shown in

Table 1. List of primers used for PCR assays

Pathogen	Target gene	oligonucleotide sequences (52-32)	Fragment (bp)	Reference
T. annulata	Tams1	GTAACCTTTAAAAACGT GTTACGAACATGGGTTT	721	D'oliveira *et al.* (1995)
T. orientalis	MPSP	CTTTGCCTAGGATACTTCCT ACGGCAAGTGGTGAGAACT	776	Ota *et al.* (2009)
A. ovis	MSP4	TGAAGGGAGCGGGGTCATGGG GAGTAATTGCAGCCAGGCACTCT	347	Torina *et al.* (2012)
A. phagocytophilum	Epank1	CTGAAGGGGGAGTAATGGG GGTAATAGCTGCCAGAGATTCC	444	Walls *et al.* (2000)

Table 2. Prevalence of *Theileria* and *Anaplasma* infection of cattle and sheep in Northeastern China

Collection sites	Host	No. of samples	Prevalence of *Theileria* spp. and *Anaplasma* spp. infection in cattle and sheep			
			T. annulata	*T. orientalis*	*A. ovis*	*A. phagocytophilum*
Hulunbeier (Inner Mongolia)	Cattle	90	0% (0/90)	2.2% (2/90)	–	0% (0/90)
Mudanjiang (Heilongjiang)	Cattle	37	0% (0/37)	5.4% (2/37)	–	0% (0/37)
Mudanjiang (Heilongjiang)	Sheep	115	–	0% (0/115)	57.4% (66/115)	0% (0/115)

Table 2. The PCR studies revealed that 2/90 (2.2%) of cattle from Inner Mongolia and 2/37 (5.4%) of cattle from Heilongjiang were positive for *T. orientalis*, while 66/115 (57.4%) of sheep from Heilongjiang were positive for *A. ovis*.

Sequencing and phylogenetic analysis

The sequence analysis for 4 isolated ToMPSP gene sequences (776bp) showed variation ranging from 87.6%–99.0% among cattle samples. BLAST analysis showed that the ToMPSP gene sequences obtained from this study shared 80%–99% identity compared to previously published sequences. Phylogenetic analysis revealed that ToMPSP gene sequences of this study were classified into 3 clades, type 1 (n=2), type 2 (n=1), and type 7 ((n=1) Fig. 1). Two samples (KY495507 and KY495509) were grouped in type 1 and closely related to isolates previously reported in cattle from China (AB571978), Thailand (AB562544) and Sri Lanka (AB701473). One sample (KY495506) was identified as belonging to type 2 clade and shared high identity with previously published sequences from Thailand (AB562533), India (EU700057), Sri Lanka (AB701452) and Vietnam (AB560823). In addition, one sample was grouped in type 7 and was closely related to isolates from China (AB571981, DQ078264) and Japan (D11046).

The results of the sequence analysis for 8 *A. ovis* MSP4 gene sequences (347bp) showed 100% nucleotide identity among the sheep samples. BLAST analysis showed that the *A. ovis* MSP4 gene obtained from Heilongjiang province shared 99% identity compared with previously published sequences from Hubei (JN572935) and Guizhou (JN572932) (Liu *et al.*, 2012). Phylogenetic analysis showed that *A. ovis* MSP4 gene in this study was clustered in type 1 together with the sequences isolated from Hubei (JN572933, JN572934, JN572935) and Guizhou (JN572932) provinces, China (Liu *et al.*, 2012).

DISCUSSION

Tick-borne protozoan and *Anaplasma* infections are economically important diseases that affect livestock worldwide (Jongejan & Uilenberg, 2004). China is an endemic area for various theileriosis and anaplasmosis (Cao *et al.*, 2013; Qiu *et al.*, 2016; Qin *et al.*, 2016). Genetic information of causative agents is critical for controlling and preventing infections caused by these diseases. This study was carried out to determine the molecular epidemiology and genetic diversity of *Theileria* spp. and *Anaplasma* spp. in the northeast region of China.

Our PCR results showed *T. orientalis* was the only *Theileria* sp. present in cattle sampled from both Inner Mongolia and Heilongjiang provinces, with the detection rates ranging from 2.2% to 5.4%. These findings are consistent with previous reports

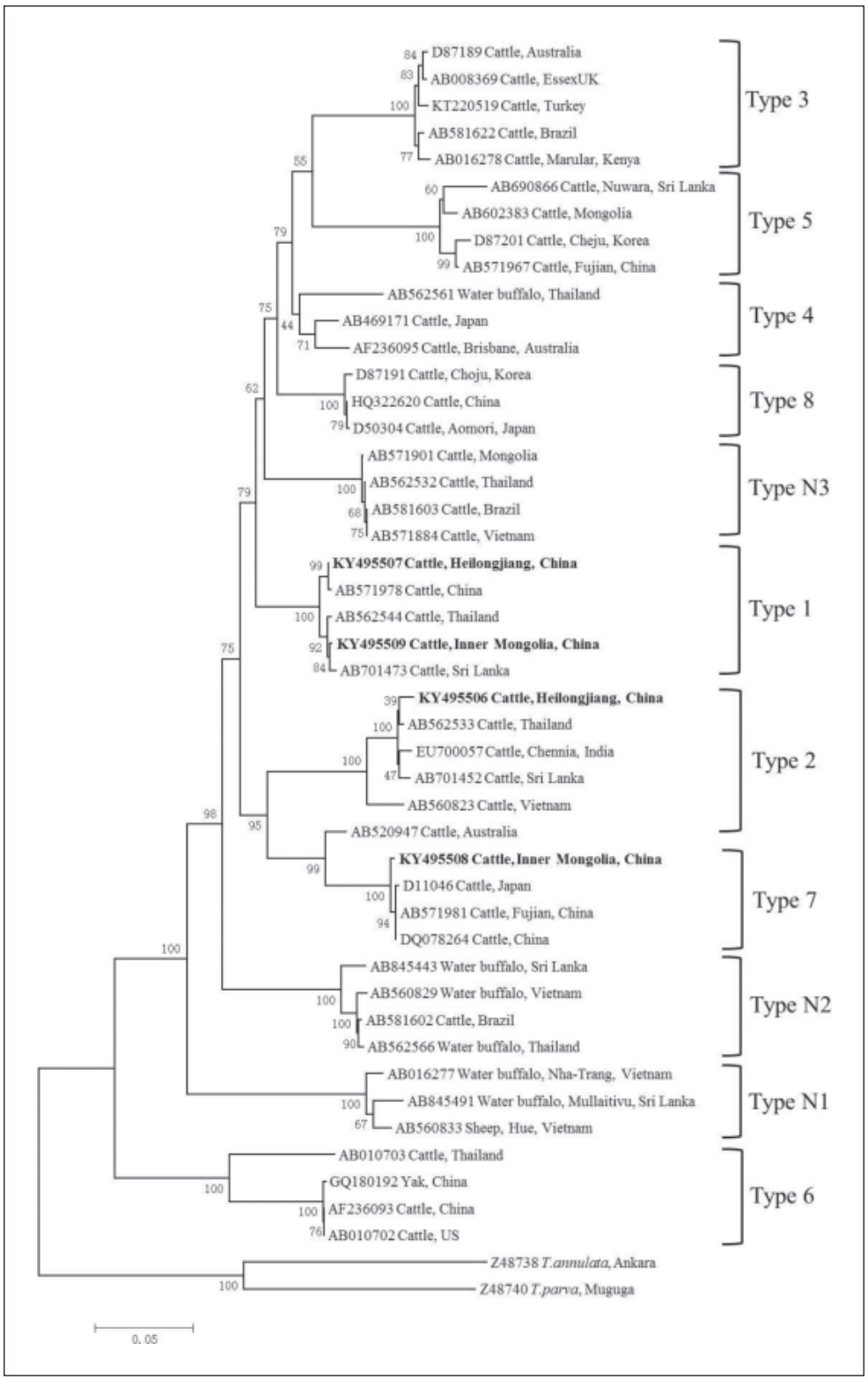

Figure 1. Phylogenetic tree analysis of *T. orientalis* MPSP gene sequences of cattle samples from northeastern parts of China (boldface letters) and other countries. The MPSP gene sequences of *T. annulata* and *T. parva* were used as outgroups.

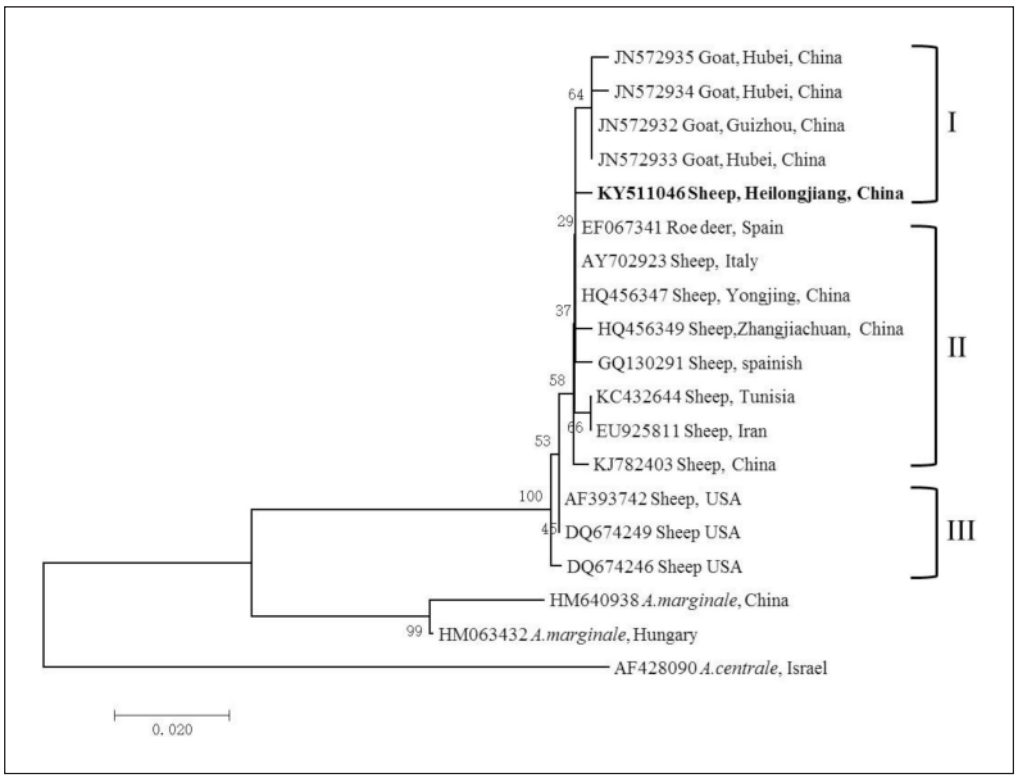

Figure 2. Phylogenetic tree analysis of *A.ovis* MSP4 gene sequences of sheep samples from northeastern parts of China (boldface letters) and other countries. The MSP4 gene sequences of *A. marginale* and *A. centrale* were used as outgroups.

showing that *T. orientalis* infection is endemic in cattle in Northeast China (Yu *et al.*, 2011). However, the prevalence rate was lower than the previous study which showed a positive rate of 13.4% in cattle (Yu *et al.*, 2011). The reason might be the samples were collected from different geographical regions. Although *T. orientalis* has additionally been reported to be present in domestic sheep of northern China (Cao *et al.*, 2013), all sheep were negative for *T. orientalis* in this study. Considering the infection of *T. orientalis* in cattle from the same endemic area, a possible reason for this is the limited sheep sample size. Therefore, a larger sample size for screening is needed to confirm these results. Moreover, this study revealed a higher infection rate (57.4%) of *A. ovis* in sheep than those previously observed in northeastern China (Qiu *et al.*, 2016). The reason might be the samples were obtained from different geographical regions. This result indicates the small ruminant industry in northeastern China faces a great threat of *A. ovis* infection.

Phylogenetic analysis revealed that *T. orientalis* MPSP sequences identified in this study were classified into three clades (type 1, type 2 and type 7). This result is in agreement with previous reports that the MPSP gene is a polymorphic antigen and shows wide diversity among different field isolates (Sivakumar *et al.*, 2014; Yokoyama *et al.*, 2011). The MPSP gene has been recognized as a useful epidemiology marker for the genotyping of the *T. orientalis* isolates in many countries (Eamens *et al.*, 2013; Zhou *et al.*, 2016; Jirapattharasate *et al.*, 2016). However, little information is available regarding the genotyping of *T. orientalis* identified in China based on the MPSP gene. Therefore, further large-scale investigations are necessary to provide essential information about the genotypic

distributions of *T. orientalis* in China. Phylogenetic analysis of the MSP4 gene sequence indicates that AoMSP4 obtained from this study together with other strains in China were classified into genotype I. This result suggests that *A. ovis* genotype I is prevalent in small ruminants in China. In addition, there are several case reports about *T. annulata* and *A. phagocytophilum* infections in other provinces of China (Yang *et al.*, 2015; Yang *et al.*, 2015; Qin *et al.*, 2016), however, none were detected in this study, which indicate that *T. annulata* and *A. phagocytophilum* may not be prevalent in this area. The tick vector species play an important role in the transmission of theileriosis and anaplasmosis, *Ixodes persulcatus* is the main tick species in the study area. Therefore, more epidemiologic study should be conducted to investigate the presence and distribution of *Theileria* spp. and *Anaplasma* spp. infections in *Ixodes* tick in the area.

In conclusion, in this study, we investigated the prevalence of tick-borne protozoan and *Anaplasma* infections in cattle and sheep from northeastern China. *A. ovis* infection was highly prevalent in sheep from Heilongjiang province. Although *T. orientalis* infection in cattle was not very high, at least three genotypes of *T. orientalis* were present in northeastern China. It is therefore recommended that appropriate management practices to be employed for the control of these tick-borne diseases in northeastern China.

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