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Original article

First molecular evidence of mixed infections of *Anaplasma* species in dogs in Henan, China

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ABSTRACT

In recent years, tick-borne diseases like anaplasmosis have become widespread worldwide threatening the health of both human and animals. Dogs play an important role in the epidemiology of several zoonotic tick-borne pathogens by acting as reservoirs. In this study, the status of *Anaplasma phagocytophilum*, *A. platys*, *A. bovis* and *A. ovis* infection were assessed in dogs in Henan, China, with PCR and phylogenetic analyses. Nested PCRs on 243 blood samples collected from dogs from different sampling sites revealed that thirty-three (13.6%) dogs were positive for one or more pathogens. The prevalence of *Anaplasma* spp. in stray dogs was 40.7% (24/59), which was much higher than that of pet dogs (4.0%, 7/175). The prevalence for *A. ovis*, *A. bovis* and *A. phagocytophilum* was 6.2%, 4.1% and 0.4%, respectively and mixed-infection of these three pathogens was found in only one stray dog (prevalence, 0.4%). None of the dogs was positive for *A. platys*. Phylogenetic analyses classified *A. phagocytophilum* into two distinct groups (East Asia and south Africa group, Europe and America group), whereas *A. ovis* and *A. bovis* showed a general classification into two groups (cluster 1 and cluster 2), respectively. The isolate (KX190783) of *A. ovis* from a stray dog fell in a clade with a human isolate from Cyprus (FJ460443) and shared 99.8% similarity with it. To the best of our knowledge, this study is the first report to identify *A. bovis* and *A. ovis* DNA in dogs in China and the mixed-infection of the three *Anaplasma* spp. (*A. phagocytophilum*, *A. bovis* and *A. ovis*) in dogs.

1. Background

Globally, vector-borne diseases pose serious public health threats, and many of them cause morbidity and mortality in dogs (Beugnet and Marié, 2009; Kelly et al., 2013). During the last decades, an increasing number and range of companion animal species have become closely associated with human beings in industrialized societies (Cito et al., 2015). Consequently, various types of companion animals, such as dogs and cats, share the same environment with humans (Fang et al., 2015; Greay et al., 2016). Considering their close association with human beings and their susceptibility to tick-borne agents, dogs can act as reservoirs for the zoonotic pathogens potentially transmitted by ticks, such as the bacteria in *Anaplasma* genus (Chomel, 2011; Lauzi et al., 2016).

Anaplasmosis is a tick-borne disease (TBD) caused by gram-negative obligate intracellular bacteria in the *Anaplasma* genus (*Rickettsiales, Anaplasmataceae*) (Dumler et al., 2001). The major species in the *Anaplasma* genus that impact animal and human health are *A. phagocytophilum*, *A. platys, A. ovis*, *A. bovis*, *A. centrale* and *A. marginale* (Liu et al., 2012b; Rar and Golovljova, 2011). Among them, the organisms with greater clinical importance in dogs include *A. phagocytophilum* and *A. platys*, which are distributed worldwide, including China (Bowman et al., 2009; Kelly et al., 2013; Lee et al., 2016; Liu et al., 2016). *A. ovis* and *A. bovis*, which are often found in ruminants have emerged as pathogens of dogs (Aquino et al., 2016; Sakamoto et al., 2010). In addition, previous studies further support the statement that *A. phagocytophilum* is a zoonotic tick-borne pathogen, while *A. platys* and *A. ovis* may have zoonotic tendency under certain conditions (Arraga-Alvarado et al., 2014; Cao et al., 2006; Chochlakis et al., 2010).

Recently, several surveys on *Anaplasma* spp. infections in dogs have been conducted across the world, and the prevalence ranges

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from 0.5% to 20.4% (Barth et al., 2011; Bowman et al., 2009; Cardoso et al., 2016; Kolo et al., 2016; Xia et al., 2011). In addition, few studies have been carried out on *Anaplasma* spp. infections in dogs in China, for instance, 62.1% (36/58) canine blood samples were found to be positive for *A. platys* by RT-LAMP in south China (Li HT et al., 2014) and the average seroprevalence of *A. phagocytophilum* was 10.05%, while the PCR-positive rate was 10.89% in dogs from ten provinces/cities of China (Zhang et al., 2012). However, there was no report on the infection of *A. ovis* and *A. bovis* in Chinese dogs until now, what's more, mixed infection of *A. phagocytophilum*, *A. platys*, *A. ovis* and *A. bovis* in dogs has never been investigated before. To provide further information on *Anaplasma* spp. epidemiology in dogs, the prevalence of these pathogens was investigated in Henan province, China.

2. Methods

2.1. Sample collection and DNA extraction

During 2013–2015, a total of 243 EDTA-K2 whole blood samples were collected from four pet hospitals (pet dogs, n = 175), four animal shelters (stray dogs, n = 59), six sheep farms (guard dogs, n = 6) and one police dog base (police dogs, n = 3) in Henan province, central China (Fig. 1). For each dog, one blood sample was collected from the jugular vein. The dogs sampled from pet hospitals and police dog base were sent to see veterinarians for unknown diseases. All the sampled stray dogs, randomly selected from animal shelters were asymptomatic. In China, almost every sheep farm likes to keep one dog for guarding their belongings, so samples from apparently healthy guard dogs were collected from six different sheep farms. No ticks feeding on dogs were found in this study.

Genomic DNA was extracted from 200 μl blood samples using a Blood DNA Extraction Kit (Lifefeng, Shanghai, China), following the manufacturer's instructions. The DNA extracted was re-suspended in TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA) and stored at −20 °C until use.

2.2. PCR amplification

Nested PCRs targeting the 16S rRNA gene were carried out to detect the presence of *A. bovis* and *A. phagocytophilum* DNA in the blood samples. During the first round, the EE1/EE2 primer pair was used as previously described (Barlough et al., 1996). The PCR products were used as templates for the second round using *A. bovis*-specific primers AB1f/AB1r, and *A. phagocytophilum*-specific primers SSAP2f/SSAP2r, which amplified a 551 bp and 641 bp fragment of 16S rRNA gene, respectively (Barlough et al., 1996; Kawahara et al., 2006). For PCR identification of *A. ovis* DNA, the major surface protein 4 (msp4) gene was amplified as described previously (de la Fuente et al., 2007). PCR reaction was carried out using *A. platys* specific primers EPLAT5/EPLAT3 amplifying a 349 bp fragment of the 16S rRNA gene (Murphy et al., 1998). To avoid false-positive results, PCR was conducted at least twice and nuclease-free water was used as negative control in all PCR reactions. DNAs positive for *A. phagocytophilum*, *A. platys*, *A. ovis* and *A. bovis*, confirmed by sequencing and preserved in our laboratory, were used as positive controls in each PCR assay. PCR products (5 μl) were analyzed by electrophoresis on a 2% agarose gel.

2.3. DNA sequencing and phylogenetic analysis

The positive PCR products were sequenced in a commercial company (GENEWYIZ, Beijing, China) by an ABI PRISM 3730 XL DNA Analyzer using a BigDye Terminator v3.1 Cycle Sequencing

Table 1
Detection of *Anaplasma* pathogens in different samples.

Species	No. tested	No. positive (%)								
			amplification primer for one pathogen			amplification primers for two pathogens				amplification primers for three pathogens
			EE1/2+ SSAP2f/r[a]	EE1/2+ AB1f/r[b]	MSP45/3[c]	EE1/2+ SSAP2f/r+ MSP45/3	EE1/2+ SSAP2f/r+EE1/2+ AB1f/r	MSP45/3+ EE1/2+ AB1f/r		EE1/2+ SSAP2f/r+ MSP45/3+EE1/2+ AB1f/r
Pet dogs	175	7(4.0)	0(0)	3(1.7)	4(2.3)	0(0)	0(0)	0(0)		0(0)
Stray dogs	59	24(40.7)	0(0)	12(20.3)	6(10.2)	0(0)	2(3.4)	3(5.1)		1(1.7)
Guard dogs	6	2(33.3)	1(16.7)	0(0)	0(0)	0(0)	0(0)	1(16.7)		0(0)
Police dogs	3	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)		0(0)
Total	243	33(13.6)	1(0.4)	15(6.2)	10(4.1)	0(0)	2(0.8)	4(1.6)		1(0.4)

[a] Primer used for amplifying of *Anaplasma phagocytophilum* 16S rRNA.
[b] Primers used for amplifying of *Anaplasma bovis* 16S rRNA.
[c] Primers used for amplifying of *Anaplasma ovis* msp4.