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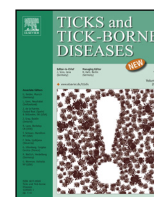

Original article

Discovery of *Rickettsia* species in *Dermacentor niveus* Neumann ticks by investigating the diversity of bacterial communities

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ABSTRACT

Ticks (*Dermacentor niveus* Neumann) were collected from Tacheng, Xinjiang Uygur Autonomous Region, and their bacterial diversity was investigated using the 16S RNA gene library method from one pooled sample. A total of 452 clones was successfully sequenced and assigned to 4 phyla. The dominant phylum was the Proteobacteria, accounting for 62.8% of all the clones of the 16S rRNA gene at the confidence level 80%. The other sequences were assigned to the phyla Bacteroidetes, Firmicutes, Actinobacteria and accounted for 13.5%, 12.4%, and 11.3%, respectively. These results provide an insight into the bacterial diversity associated with *D. niveus* ticks in the natural environment of Tacheng. They indicate the occurrence of *Rickettsia raoultii* and *Rickettsia slovaca* in *D. niveus* ticks in this area, and as a consequence, cases of TIBOLA/DEBONEL may occur (tick-borne lymphadenopathy/*Dermacentor*-borne necrosis erythema and lymphadenopathy).

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Introduction

Ticks are efficient vectors of multiple pathogens due to their interactions with a wide spectrum of vertebrate hosts during their life cycle. As a result, they have the opportunity to acquire a large array of different types of organisms that are present in the blood of these hosts. The microbial community in ticks includes viruses, bacteria, protozoa, and fungi, which serve as symbionts, commensals, and as pathogens (Anderson and Magnarelli, 2008). Multiple human pathogens can be carried by a single tick (Belongia, 2002), and exposure to a single tick may cause the risk of multiple infections. Therefore, the exploration of microbial populations from ticks might help to identify the pathogens causing human disease.

Culture-dependent (Murrell et al., 2003; Rudolf et al., 2009) techniques were applied by Sanogo et al. (2003) studying the microbial populations in ticks. In the Czech Republic, culture-dependent methods were used to identify the microorganisms from host-seeking *Ixodes ricinus* (*I. ricinus*), *Dermacentor reticulatus*, and *Haemaphysalis concinna* ticks, from which strains of medical importance were found, including *Advenella incenata*, *Corynebacterium aurimucosum*, *Microbacterium oxydans*, *M. schleiferi*, *Staphylococcus* spp., and *Stenotrophomonas maltophilia* (Rudolf et al., 2009). In recent years, the method of metagenomics has been increasingly applied for exploration of microbial communities present in complex ecosystems. The small-subunit ribosomal RNA library method based on amplifying the 16S rRNA gene directly offers an effective way to identify bacterial and archaeal diversity from environmental samples and to estimate dynamics in a complex microbial community (Pace, 1997). The 16s rRNA gene has several conserved regions shared by a large number of bacterial species and variable regions which could be used to clarify the taxonomic affinities of a wide range of taxa (Baker et al., 2003). Universal primers are designed according to the conserved regions to amplify as many bacterial species as possible, and then the clonal library of the PCR product is screened for microbial taxa. Once microbial taxa of interest are identified, further investigation might be performed to test the existence of the specific microbes efficiently. For example, an investigation on *I. ricinus* ticks collected from the Netherlands disclosed the presence of *Candidatus* Neoehrlichia mikurensis, *Rickettsia australis*, and *Borrelia* species by this method (van Overbeek et al., 2008).

Dermacentor niveus Neumann has been recorded as one of the dominant tick species at the sampling site. It is a parasite of humans

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and a wide range of livestock (Deng and Jiang, 1991; Yin et al., 2010). The city of Tacheng was selected as the sampling site, as it is the key region in bridging China, Central Asia, and Europe, with a high density of *D. niveus*, a tick species that might transmit pathogens causing widespread infectious diseases. Here, we evaluated the bacterial diversity in *D. niveus* ticks collected from Tacheng using the 16S rRNA gene library approach. This characterization of the bacterial community in *D. niveus* is a fundamental step toward understanding its role as a vector in carrying and transmitting known and yet to be discovered pathogens in these as well as in adjacent regions.

Materials and methods

Collection and identification of ticks

The field investigation was carried out in August 2008. Ticks were collected by dragging over the vegetation layer at Yumin town (82°54′ E, 46°12′ N) in Tacheng city, Xinjiang Uygur Autonomous Region.

Morphologic features were used to identify species and developmental stage of the collected ticks by an entomologist (Y. Sun). Live ticks were then stored in 70% ethanol at −80 °C until nucleic acid extraction.

PCR amplification of tick pools

A total of 83 adult ticks was pooled and disrupted in liquid nitrogen by pestles. DNA was extracted from the obtained powder using the phenol-chloroform extraction method (Wen et al., 2002). The 16S rRNA gene was amplified from the obtained DNA with the primers 16S F and 16S R (Table 1) (Zhang and Chen, 2010) in 50 μL PCR mixtures containing 200 nM of each primer, 100 mM of each dNTP, 5 μL 10× rTaq PCR buffer (Takara, Dalian, China), and 2.5U of rTaq DNA polymerase (Takara, Dalian, China). PCR conditions were as follows: one cycle at 94 °C for 5 min; 25 cycles at 94 °C for 30 s; 55 °C for 40 s; 72 °C for 90 s, and one cycle at 72 °C for 10 min. The PCR was processed using ABI GeneAmp® PCR System 9700. Agarose gel with target fragments was purified using TIANgel Midi Purification Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The purified product was then cloned to pMD-18T vector (Takara, Dalian, China) and introduced in the *E. coli* DH5α (Tiangen, Beijing, China). A single colony was cultured and sequenced on an Applied Biosystems 3730XL capillary sequencer with the vector primers M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGACC-3′).

Sequence analysis

The clones were sequenced from both ends using the primers M13F and M13R of the vector by standard techniques. Low-quality parts and vector sequences of each sequence were trimmed off. Then, the 2 sequences from one single clone were assembled from both ends to one sequence. Primer sequences of each obtained assembled sequence were cut off to avoid affecting the results. Thereafter, the obtained sequences were analyzed by the "Classifier" program (version 2.6) of Ribosomal Database Project II (Cole et al., 2005). The Classifier algorithm returns a confidence value with which a 16S rRNA gene sequence can be assigned to OTUs (genus and higher) that is represented by a set of sequences, based on the number of times, out of 100 trials, that random subsets of the query sequence match sequences assigned to that taxon. The algorithm also returns the name of the taxon to which the sequence was most often assigned in those 100 trials.

Identification of Rickettsia and phylogenetic analysis

After having evidence of the occurrence of *Rickettsia*, we performed a PCR with *Rickettsia*-specific PCR primers (Table 1) for the *ompB* and *gltA* genes for further identification of *Rickettsia* (Fernandez de Mera et al., 2009; Roux et al., 1997) using the previously obtained DNA as target. The PCR products were analyzed on a 1.5% agarose gel. Fragments of the right size were then purified, cloned to pMD-18T vector (Takara, Dalian, China), and introduced in the *E. coli* DH5α. A single colony was cultured and sequenced. The clones were sequenced by standard techniques, and clean data were obtained as previously described. Blast search were then performed against nucleotide collection (nr/nt) database on the website of NCBI (http://www.ncbi.nlm.nih.gov/).

Phylogenetic analysis was performed on *ompB* and *gltA* gene sequences, respectively, with the gene sequences of *Rickettsia* species obtained from GenBank as references. *Rickettsia typhi* strain Wilmington was selected as outgroup. The phylogenetic analysis of the remaining part was performed with Mega 5 software v5.1 (Tamura et al., 2011). The alignment was conducted by ClustalW (1.6) (Thompson et al., 1994). The gap opening penalty was 15 and the gap extension penalty was 6.66. The DNA Weight Matrix was ClustalW, and the transition weight was 0.5. Phylogenetic analysis was performed by the maximum likelihood method with 1000 bootstrap replications.

Results

Bacterial diversity

A total of 500 clones of the fragment amplified with the primers 16S 27F and 16S 1492R was sequenced from both ends, and 452 high-quality assembled sequences were obtained (GenBank nos. KJ453898–KJ454349). The details of the assignments are shown in Fig. 1. The dominant phylum was the Proteobacteria, accounting for 62.8% of all the clones of the 16S rRNA gene at the confidence level 80%. The other phyla were assigned to the Bacteroidetes, the Firmicutes, and the Actinobacteria and accounted for 13.5%, 12.4%, and 11.3%, respectively. The tick-borne pathogens *Rickettsia raoultii* and *Rickettsia slovaca* were found to exist in *D. niveus* ticks. Opportunistic pathogens like *Pseudochrobactrum* and *Massilia* were also found in moderate frequencies. Also environmental soil microorganisms such as *Sphingobium* and *Acinetobacter* were detected.

Identification of Rickettsia and phylogenetic analysis

Two clones were shown to belong to the genus *Rickettsia* which is recognized as a medically important arthropod-vectored taxon of pathogens. The closest *Rickettsia* species was *Rickettsia raoultii* strain Khabarovsk (ref|NR_043755.1|) with 2 different base pairs resulting from BlastN analysis of the two 16S rRNA clones initially identified to belong to the genus *Rickettsia*. Further identification of *Rickettsia* targeting at *ompB* and *gltA* genes produced the amplicons of 576 bp (618 bp with primer) and 1136 bp (1178 bp with primer), respectively. Clones of each gene were obtained as previously described.

All the obtained 10 *gltA* (JQ664720–JQ664729) sequences were most similar to that of the *R. raoultii* strain Khabarovsk (GenBank accession no. DQ365810). One of the obtained partial 11 *ompB* gene sequences (GenBank accession no. JQ320350) was most similar to *R. slovaca* (GenBank accession no. AF123723) with 98.8% identity while the other 10 (GenBank accession no. JQ320340–JQ320349) displayed 99.7–99.8% identity with the *R. raoultii* strain Khabarovsk.

For the phylogenetic analysis (Fig. 2), 10 obtained sequences of the rickettsial *gltA* gene clustered close to *R. raoultii*. Also 10 of the

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