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PCR-DGGE test for direct identification of intestinal bacterial flora in blood feeding ticks in China

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Abstract Ticks are vectors of diseases that affect humans and animals worldwide. In current study, the intestinal bacterial flora associated with the blood feeding ticks (*Haemaphysalis flava*, *Haemaphysalis longicornis*, *Rhipicephalus haemaphysaloides*, *Boophilus microplus* and *Dermacentor sinicus*) were analyzed using polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) and then sequenced. The five ticks were collected from cattle, dog, hedgehog and goats in Fujian, Shandong, Henan, Jiangxi, Hunan, Shanxi and Guangxi provinces, China. Our results show that nine distinct DGGE bands were found using PCR-DGGE method. Sequences analyses indicated that they belonged to *Rickettsia peacockii*, *Rickettsia raoultii*, *Rickettsia helvetica*, *Rickettsia slovaca*, *Rickettsia tarasevichiae*, *Coxiella* sp., *Erwinia* sp., *Klebsiella pneumoniae* and *Pseudomonas aeruginos*. The present results indicate that zoonotic pathogens are present in ticks in many provinces of China. This useful information will aid in the epidemiology of tick-borne zoonotic diseases in China as well as in raising awareness to avoid tick bites is an important measure to prevent the infection and transmission of zoonotic pathogens.

INTRODUCTION

Ticks, as obligate blood-sucking ectoparasites, attack a broad range of animals and humans, and they are considered second only to mosquitoes as vectors of human disease. Ticks and tick-borne diseases (TBD) are a/an growing/increasing problem affecting human health around the world (Liu *et al.*, 2014; Imhoff *et al.*, 2015). Ticks are also the most important ectoparasites of livestock worldwide, and are responsible for severe economic losses due to the ability to transmit viruses, bacteria, rickettsiae, helminthes and protozoans, all of which are able to cause damage to livestock production and health (de la Fuente & Contreras, 2015).

There are an estimated about 900 species of ticks belonging to three families: Argasidae, Ixodidae, and Nuttalliellidae (Dantas-Torres *et al.*, 2012). In China, more than 100 species of the following genera have been identified: *Argas*, *Carios*, *Ornithodoros*, *Amblyomma*, *Anomalohimalaya*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus* (Chen *et al.*, 2010). Some of these tick species carry or transmit one or more infectious pathogens, causing severe zoonotic diseases. In China, the most commonly observed human tick-borne diseases are reportedly Lyme disease, tick-borne encephalitis, Crimean-Congo hemorrhagic fever, Q fever, tularemia, spotted fever and severe fever with Thrombocytopenia Syndrome (Gao *et al.*, 2007; Wu *et al.*, 2013). However, tick associated with pathogens or diseases are still underestimated because of the complex distribution and the large diversity of tick species in China. Given that Next-generation sequencing tends to be expensive, polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) has proven to be a cheap and useful tool to

analyze the intestinal microbial diversity in many animals (Ariefdjohan *et al*., 2010; Li *et al*., 2012) and has also been extensive used to detect the microbial populations of ticks (Moreno *et al*., 2006; Halos *et al*., 2006). So far, In spite of their significance as pathogens, little information is available about identification of intestinal bacterial flora in ticks using DGGE method (Schabereiter *et al*., 2003; Van Overbeek *et al*., 2008; Tveten *et al*., 2013; Tveten *et al*., 2013; Xu *et al*., 2015).

The objectives of the present study were investigate the bacterial communities in five blood feeding ticks using PCR-DGGE method. The results of the present investigation have implications for the ongoing control and prevent the infection and transmission of zoonotic pathogens in humans and animals in China.

MATERIALS AND METHODS

Collection of ticks and DNA extraction

Adult ticks were collected from cattle, dog, hedgehog and goats in Fujian, Shandong, Henan, Jiangxi, Hunan, Shanxi and Guangxi provinces, China. These ticks represent *Haemaphysalis flava*, *Haemaphysalis longicornis*, *Rhipicephalus haemaphysaloides*, *Boophilus microplus* and *Dermacentor sinicus*). Feeding ticks were individually picked from cattle and goats using sterile tweezers. After all ticks were rinsed by 70% ethanol for about 2 min, placed individually into sterile tubes and then grind. Total genomic DNA was isolated from individual tick (grinding fluid) using DNeasy bacteria kit according to manufacturers' protocol (TransGen Corporation, Beijing, China). The DNA was eluted with 100 µl elution buffer. DNA was analyzed by $10g.L^{-1}$ agarose gels with a molecular weight standard and stored at -20°C until further use.

PCR amplification

Primer 318f (5'-CCTACGGGAGGCAGCAG-3') and 907r (5'-CCCCGTCAATTCATTTGAGTTT-3') were used to amplify V3 regions of gene fragments of 16S rDNA. Reaction mixtures of 50 µl contained 4 µl of genomic DNA, 2 µl of each primer, 25 µl of 2×Taq mix, DNA samples were amplified with the following steps: initial denaturation for 5 min at 94°C, denaturation 30 cycles for 30s at 95°C, annealing for 1min at 59°C, primer extension for 45s at 72°C and final extension for 7 min at 72°C. A negative control was included in all PCR-DGGE experiments. Presence of PCR products were confirmed by electrophoresis on 1.5% agarose gels stained with ethidium-bromide in 1×TAE buffer using a 100 bp DNA mass ladder. Gels were visualized and photographed by UV transillumination.

Denaturing gradient gel electrophoresis

The PCR amplicons were subjected to DGGE with a 30 ~ 60% linear denaturing gradient of urea and formamide in a 6% acrylamide gels according to the manufacturer's instructions (JunYi, Beijing, China). 5 µl of PCR product along with 1 µl of 6×loading buffer was loaded in each lane. Electrophoresis was performed in 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, PH8.0 with NaOH) for 4 hours at a constant voltage of 120V at 60°C. The gels stained with argentation before they photographed by UV transillumination, and then DGGE band recoverd.

Second amplification

The selected dominant bands were excised from the gel and eluted in 20 µl sterile water at 4°C overnight and then frozen at –20°C. 3 µl of DNA was used as a template and re-amplified with the forward primer $341f_{GC}$ (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3') and518r (5'-ATTACCGCGGCGCTGG-3') by following the program described previously. Each PCR product was also subjected to DGGE analysis to confirm where the bands have been eluted. Clone library construction for sequencing. Purified PCR products were then cloned into the PMD18-T vector (Takara, Dalian, China) for sequencing and introduced into Escherichia coli DH5α by transformation, according to the protocol provided by the manufacturer. The transformed cells were plated onto LB medium (1.0% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1.0% NaCl, 1.5% Bacto agar, pH

7.0) containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galgacto-pyrano-side: 0.1mM) to identify white-colored recombinant colonies. The clones were selected for sequencing. All sequencing was conducted at the Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. in China.

DGGE profile analysis

Each sequence was compared to sequences of known bacterial species in the BLAST database. The fringerprints of the DGGE profile were analyzed using the Quantity One analysis software version 4.6.2. Comparisons between different animals and different regions were performed. The number of bands in every DGGE profile was determined as an indicator of richness.

RESULTS AND DISCUSSION

Nine different bacterial strains (codes: 1-9) were isolated by amplification of 16s DNA with specific primers. The sequence blasting results of those bacteria in Genbank showed that they belonged to *Rickettsia peacockii*, *Rickettsia raoultii*, *Rickettsia helvetica*, *Rickettsia slovaca*, *Rickettsia tarasevichiae*, *Coxiella* sp., *Erwinia* sp., *Klebsiella pneumoniae* and *Pseudomonas aeruginos* strain. The 16s DNA gene sequences have more than 97% identity to previously published sequences in Genbank (Table 1). These nine bacteria were found from all ticks. In order to know the identity of intestinal flora in ticks, the bands cut from DGGE gel profiles were sequenced and the results are displayed in Table 1. The all bands showed more than 97% similarity with known sequences in the BLAST database (Table 1). Among the detected DGGE bands, six bacteria were detected in two ticks (*Rhipicephalus haemaphysaloides* and *Boophilus microplus*). *Dermacentor sinicus* only were detected two bacteria. Interestingly, band 3 and 4 were detected from *Rhipicephalus haemaphysaloides* and *Boophilus microplus*, and band 6 and 7 was only detected from *Haemaphysalis longicornis*, and band 9 was only detected from *Haemaphysalis flava*. Their sequences blasting analysis shows that they belonged to *Rickettsia peacockii* (Band 1), *Klebsiella pneumoniae* (Band 2), *Rickettsia tarasevichiae*, *Coxiella* sp. (Band 3), *Rickettsia raoultii* (Band 4), *Rickettsia helvetica* (Band 5), *Rickettsia slovaca* (Band 6), *Erwinia* sp. (Band 7) and *Pseudomonas aeruginos* (Band 8) and *Candidatus Rickettsia* (Band 9).

Common bacteria identified in ticks have been reported in many countries and regions (Ghosh & Nagar, 2014; Chikeka & Dumler, 2015; Narasimhan & Fikrig, 2015). Our findings are thus in agreement with these previous reports. The present results show that many bacteria in ticks can be detected by DGGE method. The DGGE method has been very popular for bacteria detection in ticks because The DGGE has the advantages such as reproducibility, rapidity, reliability and allows screening of multiple samples (Kušar *et al.*, 2012; Xu *et al.*, 2015). So, the

Table 1. The 16s DNA gene sequences have more than 97% identity to previously published sequences in Genbank

Band	Accession	Nearest march	Identity
1	NR118837.1	Rickettsia peacockii strain Skalkaho 16S ribosomal RNA gene	99%
2	KR092085.1	Klebsiella pneumoniae strain SRP2 16S ribosomal RNA gene	99%
3	KC776318.1	Uncultured Coxiella sp. clone CYP-1 16S ribosomal RNA gene	100%
4	KJ410261.1	Rickettsia raoultii isolate BL029-2 16S ribosomal RNA gene	98%
5	KR150777.1	Rickettsia helvetica strain 43Tr 16S ribosomal RNA gene	100%
6	KJ410262.1	Rickettsia slovaca isolate TC250-17 16S ribosomal RNA gene	97%
7	FM161470.1	Erwinia sp. 01WB03.3-26 partial 16S rRNA gene, strain 01WB03.3-26	99%
8	KJ081975.1	Pseudomonas aeruginosa strain NIOPACL17 16S rRNA gene	98%
9	KR150778.1	Candidatus Rickettsia tarasevichiae strain 14Ip 16S ribosomal RNA gene	98%

present study, we used DGGE method to detect bacteria in ticks. In this study, the *Rickettsia peacockii*, *Rickettsia raoultii*, *Rickettsia helvetica*, *Rickettsia slovaca*, *Rickettsia tarasevichiae*, *Coxiella* sp., *Erwinia* sp., *Klebsiella pneumoniae* and *Pseudomonas aeruginos* strain were detected in ticks. These results have indicated that these bacteria are considered as predominant bacteria in ticks in China, consistent with previous studies (Gao *et al*., 2010; Liu *et al*., 2013; Sun *et al*., 2015).

In summary, DGGE profiling was used to identify microorganisms associated with five ticks. Sequences analyses indicated that they belonged to *Rickettsia peacockii*, *Rickettsia raoultii*, *Rickettsia helvetica*, *Rickettsia slovaca*, *Rickettsia tarasevichiae*, *Coxiella* sp., *Erwinia* sp., *Klebsiella pneumoniae* and *Pseudomonas aeruginos*. The present results indicate that zoonotic pathogens are present in ticks in many provinces of China. This useful information will aid in the epidemiology of tick-borne zoonotic diseases in China as well as in raising awareness to avoid tick bites is an important measure to prevent the infection and transmission of zoonotic pathogens.

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REFERENCES

Ariefdjohan, M.W., Savaiano, D.A. & Nakatsu, C.H. (2010). Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutrition Journal* **9**: 23.

Chen, Z., Yang, X.J., Bu, F.J., Yang, X.H., Yang, X.L. & Liu, J.Z. (2010). Ticks (Acari: Ixodoidea: Argasidae, Ixodidae) of China. *Experimental and Applied Acarology* **51**: 393-404.

Chikeka, I. & Dumler, J.S. (2015). Neglected bacterial zoonoses. *Clinical Microbiology and Infection* **21**: 404-415.

Dantas-Torres, F., Chomel, B.B. & Otranto, D. (2012). Ticks and tick-borne diseases: a one health perspective. *Trends Parasitoogy* **28**: 437-446.

de la Fuente, J. & Contreras, M. (2015). Tick vaccines: current status and future directions. *Expert Review of Vaccines* 2015 [Epub ahead of print].

Gao, X., Nasci, R. & Liang, G. (2010). The neglected arboviral infections in mainland China. *PLoS Neglect Tropical Diseases* **4**: e624.

Halos, L., Mavris, M., Vourch, G., Maillard, R., Barnouin, J., Boulouis, H.J. & Vayssier-Taussat, M. (2006). Broad-range PCR-TTGE for the first-line detection of bacterial pathogen DNA in ticks. *Veterinary Research* **37**: 245-253.

Imhoff, M., Hagedorn, P., Schulze, Y., Hellenbrand, W., Pfeffer, M. & Niedrig, M. (2015). Review: Sentinels of tick-borne encephalitis risk. *Ticks and Tick Borne Diseases* **6**: 592-600.

Kušar, D. & Avguštin, G. (2012). Optimization of the DGGE band identification method. *Folia Microbiology* **57**: 301-306.

Li, S., Sun, L., Wu, H., Hu, Z., Liu, W., Li, Y. & Wen, X. (2012). The intestinal microbial diversity in mud crab (*Scylla paramamosain*) as determined by PCR-DGGE and clone library analysis. *Journal of Applied Microbiology* **113**: 1341-1351.

Liu, L., Li, L., Liu, J., Hu, Y., Liu, Z., Guo, L. & Liu, J. (2013). Coinfection of Dermacentor silvarum olenev (acari: ixodidae) by *Coxiella*-Like, *Arsenophonus*-like, and *Rickettsia*-like symbionts. *Applied and Environment Microbiology* **79**: 2450-2454.

Liu, Q., He, B., Huang, S.Y., Wei, F. & Zhu, X.Q. (2014). Severe fever with thrombocytopenia syndrome, an emerging tick-borne zoonosis. *Lancet Infectious Diseases* **14**: 763-772.

Moreno, C.X., Moy, F., Daniels, T.J., Godfrey, H.P. & Cabello, F.C. (2006). Molecular analysis of microbial communities identified in different developmental stages of *Ixodes scapularis* ticks from Westchester and Dutchess Counties, New York. *Environment Microbiology* **8**: 761-772.

Schabereiter, G.C., Lubitz, W. & Rölleke, S. (2003). Application of broad-range 16S rRNA PCR amplification and DGGE fingerprinting for detection of tick-infecting bacteria. *Journal of Microbiology Methods* **52**: 251-260.

Sun, J., Lin, J., Gong, Z., Chang, Y., Ye, X., Gu, S., Pang, W., Wang, C., Zheng, X., Hou, J., Ling, F., Shi, X., Jiang, J., Chen, Z., Lv, H. & Chai, C. (2015). Detection of spotted fever group Rickettsiae in ticks from Zhejiang Province, China. *Experimental and Applied Acarology* **65**: 403-411.

Tveten, A.K., Riborg, A. & Vadseth, H.T. (2013). DGGE identification of micro-organisms associated with *Borrelia burgdorferi* Sensu Lato-or *Anaplasma phagocytophilum*-infected *Ixodes ricinus* ticks from northwest Norway. *International Journal of Microbiology* **2013**: 805456.

Tveten, A.K. & Sjåstad, K.K. (2011). Identification of bacteria infecting Ixodes ricinus ticks by 16S rDNA amplification and denaturing gradient gel electrophoresis. *Vector Borne Zoonotic Diseases* **11**: 1329-1334.

Van Overbeek, L., Gassner, F., Van Der Plas, C.L., Kastelein, P., Nunes-da Rocha, U. & Takken, W. (2008). Diversity of Ixodes ricinus tick-associated bacterial communities from different forests. *FEMS Microbiology Ecology* **66**: 72-84.

Wu, X.B., Na, R.H., Wei, S.S., Zhu, J.S. & Peng, H.J. (2014). Distribution of tick-borne diseases in China. *Parasties & Vectors* **6**: 119.

Xu, X.L., Cheng, T.Y., Yang, H. & Yan, F. (2015). Identification of intestinal bacterial flora in Rhipicephalus microplus ticks by conventional methods and PCR-DGGE analysis. *Experimental and Applied Acarology* **66**: 257-268.