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# AN UPDATE ON THE *IXODES RICINUS* MICROBIOME

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**ABSTRACT**

*Ixodes ricinus* is vector in the transmission of many important infectious diseases in human and animals. There is still minimal information available on the bacterial agents associated with ticks found in Slovakia. We performed a survey of the bacterial communities associated with *Ixodes ricinus* collected from forest area near a great city aglomeration. Bacterial 16S rRNA hypervariable region amplicon libraries prepared from the *Ixodes ricinus* females were sequenced on Illumina MiSeq platform. We detected a total of 107 bacterial genera and order Clostridiales and class Bacilli (with more than 100 reads). Dominant taxa included the tick endosymbionts e.g. spotted fever group (*Rickettsia* 21998 reads) and *Coxiella* (10715 reads). Second dominant species was mycoplasma *Spiroplasma ixodetis* (9554 reads).
Dominant enviromental soil bacteria were *Pseudomonas* (6741 reads), *Nocardiodes* (4753 reads), *Brevundimonas* (2797 reads), *Devosia* (2797 reads) *Aureimonas* (1398 reads), *Actinomycetospora* (964 reads), *Terrimonas* (962 reads), *Pedobacter* (1139 reads), *Methylobacterium* (956 reads), *Rhodococcus* (945 reads), *Williamsia* (832 reads), *Rhizobium* (787 reads), *Mesorhizobium* (631 reads), *Rhizobium* (602 reads) and *Spirosoma* (486 reads) .
Firmicutes were only 280 reads with 151 reads of Clostridia and 128 reads of Bacilli (include *Staphylococcus* and *Bacillus* ). The cultivation and Maldi Tof analysis revealed seven species of coagulase negative staphylococci (CoNS) i.e., *Staphylococcus epidermidis, S. capitis, S.haemolyticus, S.hominis, S. pasteuri, S.chromogenes, S.warneri* and *Micrococcus luteus*. Antibiotic resistant CoNS with *msrA* and *blaZ genes* were detected. Five species of bacilli i.e. *Bacillus cereus, B. licheniformis , B. thuringiensis* and *B. mycoides* and two species of *Brevibacillus* i.e. *B. parabrevis* and *B. agri* were detected by Maldi Tof, also. The results of the next generation sequencing revealed a new look at the complexity of the *Ixodes ricinus* microbiome.

**Keywords:** *Ixodes ricinus*, bacteria, NGS, Maldi tof, *Staphylococcus*, antibiotic resistance

## INTRODUCTION

Ticks are important vectors of pathogens affecting humans and animals worldwide. They do not only carry pathogens but diverse commensal and symbiotic microorganisms are also present in ticks. A molecular screening for tick-borne pathogens and endosymbionts in *Ixodes ricinus*, *Dermacentor reticulatus* and *Haemaphysalis inermis* from Slovakia revealed the presence of *Rickettsia* spp., *Coxiella burnetii*, *Coxiella-like* and *Francisella-like* microorganisms (**Špitalská** *et al.* 2018). **Bielawska-Drózd** *et al.* (2016) detected the presence of Coxiella burnetii in 1,33 % of Poland ticks 1,33 % by real time PCR of IS1111 gene sequence. **Egyed** and **Makrai** (2014) found that the most frequent bacteria isolated from three tick species were *Staphylococcus* (18.1%) and *Bacillus* (7.8%). Coagulase-negative staphylococci (CoNS) have become increasingly recognized as important agents of skin infections (**Sader** *et al.* 2010). However there are no data available about antimicrobial resistance of staphylococci isolated from ticks.
In recent years the number of studies, mostly from USA, Australia and China, using NGS to investigate the microbial diversity and composition of ticks has expanded (**Greay** *et al.* 2018). However in Europe only from France (**Vayssier-Taussat** *at al.* 2013) and northern Italy (**Carpi** *et al.* 2011) NGS of *Ixodes ricinus* was used. The aim of this study was to describe the microbiome data of *Ixodes ricinus* microbiome by next generation sequencing and by cultivation experiments in Slovakia.

## MATERIAL AND METHODS

### Ticks, DNA isolation and bacterial cultivation

Ticks of *Ixodes ricinus* ticks were collected by flagging near a city agglomeration characterised by the presence of sylvatic deciduous forest. Females of ticks were sampled in 96% ethanol, rinsed in saline and homogenised. DNA was extracted by commercial kit (DNAeasy tissue kit, Qiagen, Hilden, Germany). Homogenised ticks were cultivated overnight in Nutrient broth (Oxoid Ltd, UK) at 37°C. Broth cultures were inoculated on Mannitol Salt agar (Oxoid Ltd, UK) and on blood agar (containing 10 % defibrinated sheep blood). Suspect colonies were detected by MALDI-TOF biotyper (Bruker Daltonics).

### Next generation sequencing

Bacterial 16S rDNA were amplified using primers 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1062R (5´-ACA GCC ATG CAG CAC CT-3´) to amplify V1-V6 hypervariable regions (**Ghyselinck** *et al*. 2013). The reaction mixture (25 µl) contained 1.25 U thermostable DNA polymerase (Cheetah Hot Start Taq Polymerase; Biotium, Hayward, California, USA), 1× buffer supplied with the polymerase, 1,5 mmol.l $^{-1}$ $MgCl_2$, 340 µmol.l $^{-1}$ dNTP (Applied Biosystems) and 300 nmol.l $^{-1}$ each primer. PCR was carried out in a Veriti thermal cycler (Applied Biosystems, Foster City, California, USA) using a program for 16S region consisting of initial denaturation at 94 °C for 2 min, 35 cycles (denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 2 min) and final extension at 72 °C for 10 min. Amplified products were analysed by agarose gel electrophoresis.
Products of PCR were pooled and purified by QIAquick PCR Purification Kit (Qiagen). Purified PCR products were diluted to equimolar concentration suitable for library preparation and were used as template for library preparation using transposon based Nextera Library preparation kit (Illumina, San Diego, CA, USA) according to standard protocol. Samples were analysed using paired end sequencing on Illumina MiSeq sequencing system (Illumina) in the University Science Park (Komenského University, Bratislava). Sequencing data were imported into CLC Genomics Workbench Version 7.5 (Qiagen). Each sequence of sample were marging and trimming. Limit of trimming using quality score was set to 0.001 and reads shorter than 100 nucleotides were discarded. Reads were identified based on their homology to reference 16S rRNA genes in NCBI

database using Basic Local Alignment Search Tool (BLAST) (**Altschul** *et al*. 1990). BLAST results were exported to MEtaGenome ANalyzer (MEGAN V5) (**Huson** *et al*., 2011).

**Minimal inhibitory concentrations (MIC) and PCR**

Susceptibility (MIC) was determined by Miditech (Bratislava) colourimetric broth microdilution method (http://www.eucast.org/), using ampicillin (AMP), oxacillin (OXA), moxifloxacin (MFX), erythromycin (ERY), clindamycin (CLI), linezolid (LND), tetracycline (TTC), chloramphenicol (CMP), trimethoprim (TRI), rifampicin (RIF) and teicoplanin (TEC). The presence of antibiotic resistance genes *mecA* and *mecC* (meticillin resistance), *blaZ* (beta-lactamase), *ermC* (macrolide resistance), *msrA* (efflux), *dfrS* (trimethoprim resistance) were determined by PCR (**Kmet** *et al*. 2018).

**RESULTS AND DISCUSSION**

NGS data (154 995 sequences) were analyzed to identify relative abundance of microorganisms by phylum down to genus levels. Proteobacteria were in greatest abundance, followed by Actinobacteria, Tenericutes and Bacteroidetes. The *Ixodes ricinus* mixed DNA sample showed sequence homology with 107 bacterial genera and order Clostridiales and class Bacilli (with more than 100 reads).

Figure 1 shows the occurrence of individual species of bacteria in the microbiome *Ixodes ricinus*, the size of which represents the percentage of individual readings. Dominant taxa include the tick endosymbionts e.g. spotted fever group (*Rickettsia* 21998 reads) and *Coxiella* (10715 reads). Second dominant species was mycoplasma *Spiroplasma ixodetis* (9554 reads). Dominant enviromental soil bacteria were *Pseudomonas* (6741 reads), *Nocardiodes* (4753 reads), *Brevundimonas* (2797 reads), *Devosia* (2797 reads) *Aureimonas* (1398 reads), *Actinomycetospora* (964 reads), *Terrimonas* (962 reads), *Pedobacter* (1139 reads), *Methylobacterium* (956 reads), *Rhodococcus* (945 reads), *Williamsia* (832 reads), *Rhizobium* (787 reads), *Mesorhizobium* (631 reads), *Rhizobium* (602 reads) and *Spirosoma* (486 reads). Firmicutes were only 280 reads with 151 reads of Clostridia and 128 reads of Bacilli (include *Staphylococcus* and *Bacillus*).

In the largest group causing a spotted fever group, there are 26 species of rickets (**Parola** *et al*. 2013). Typical symptoms of rickettsiosis include fever, rash, and headache. Spiroplasmas are helical mycoplasmas that infect plants and/or arthropods and may be pathogenic or commensal. A previous study in Slovakia reported an overall prevalence of *Spiroplasma ixodetis* of 3% in *Ixodes ricinus* (**Bell-Sakyi** *et al.* 2015). *Williamsia* spp. have been isolated from immunocompromised patients with diabetes mellitus, as well as in elderly patients (**Keikha** 2018). *Rhodococcus*, *Staphylococcus* and *Pseudomonas* were detected in ixodid ticks (**Rudolf** *et al*. 2009). Non-fermenting Gram-negative *Brevundimonas* spp. bacteraemia was detected in seventeen individual cases (**Ryan**, **Pembroke** 2018). *Aureimonas altamirensis* is an aerobic Gram-negative aerobe related to *Brucella* species, which is a potential opportunistic pathogen of humans (**Eshaghi** *et al.* 2015).

It remains uncertain whether ubiquitous bacteria associated with soil, plants and skin that are frequently reported in NGS studies of ticks are contaminants from environmental or host sources, or whether they are genuinely associated with the tick microbiome (**Greay** *et al*. 2018). The cultivation experiments showed the presence of staphylococci and bacilli in ethanol sterilised ticks. Maldi tof analysis revealed seven species of coagulase negative staphylococci i.e., *S.epidermidis* and *S. capitis*, *S.haemolyticus* and *S.hominis*, *S. pasteuri*, *S.chromogenes*, *S.warneri* and *Micrococcus luteus*. Five species of bacilli i.e. *Bacillus cereus*, *B. licheniformis* , *B. thuringiensis* and *B. mycoides* and two species of *Brevibacillus* i.e. *B. parabrevis* and *B. agri* were detected also.

Surprisingly majority CoNS were resistant to erythromycin, some staphylococci were resistant to ampicillin and oxacillin, rarely were resistant to trimethoprim and teicoplanin. However only *blaZ* and *msrA* genes were detected in selected coagulase negative staphylococci from *Ixodes ricinus* (Table 1). The *mecA* or *mecC* genes in phenotype positive meticillin resistant CoNS was not detected, probably due to low level of oxacillin MIC90. Antibiotic resistant coagulase-negative staphylococci can be transmitted from ticks to humans. This study indicates an important role of ixodid ticks as a indicators of antibiotic resistant staphylococci with *msrA* and *blaZ* genes. There is possible a direct relationship between ixodid ticks and their hosts of small mammals, which also carried erythromycin and oxacillin resistant staphylococci (**Kmet** *et al.* 2018).

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**Figure 1** The occurrence of individual genera of bacteria in the microbiome *Ixodes ricinus* (the size represents the individual readings)

**Table 1** Antibiotic resistance of selected coagulase negative staphylococci from *Ixodes ricinus*

| Species | Phenotype/Intepretation | PCR |
|---|---|---|
| *S. epidermidis* | ERY | *msrA* |
| *S. epidermidis* | AMP, ERY | *msrA, blaZ* |
| *S. chromogenes* | AMP, ERY, TEC | *msrA, blaZ* |
| *S. haemolyticus* | OXA, ERY, TMP, **MRCoNS** | *msrA* |
| *S. haemolyticus* | OXA, ERY, TEC, TMP, **MRCoNS** | *msrA* |
| *S. pasteuri* | ERY | *msrA* |
| *S. warneri* | ERY | *msrA* |

## CONCLUSION

The results of the next generation sequencing and cultivation revealed a new look at the complexity of the tick microbiome. Proteobacteria were in greatest abundance, followed by Actinobacteria, Tenericutes and Bacteroidetes.. Dominant taxa included the tick endosymbionts (*Rickettsia* and *Coxiella*), *Spiroplasma ixodetis* and environmental soil bacteria. However, only a minor part of the cultivable *Staphylococcus* and *Bacillus* were detected. Antibiotic resistant CoNS with *msrA* and *blaZ* genes were detected from ixodid ticks, which appear to be useful indicators of antibiotic resistance in the environment.

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