Original Article

Metagenomics Characterization of *Ixodes ricinus* Intestinal Microbiota as Major Vector of Tick-Borne Diseases in Domestic Animals

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(Received 14 Feb 2023; accepted 11 Jun 2023)

Abstract

Background: Understanding the microbiota of disease vectors can help for developing new strategies to prevent the transmission of vector pathogens. *Ixodes ricinus* is one of the most notorious tick vectors with increasing importance in Iran and other parts of the world while there is limited data on its microbiota. This study aimed to use metagenomics for identifying the *I. ricinus* tick's microbiota of Iran.

Methods: A total of 39 adult ticks were collected from Mazandaran (21 females), Gilan (17 females), and Golestan (1 male). Five tick pools prepared from 39 adults of *I. ricinus* were subjected to metagenomics analysis. The data were analyzed by targeting the V6 region of the 16S rRNA gene by Illumina 4000 Hiseq sequencing.

Results: Among hundreds of intestinal microbiota identified by metagenomics, various pathogenic microorganisms distributed in 30 genera and species including those responsible for tick-borne diseases resided in the genera *Coxiella*, *Rickettsia*, and *Burkholderia* were found.

Conclusion: Our results indicated that metagenomics identifies bacteria genera and species which cannot be easily recognized by routine methods. The presence of such pathogenic bacteria indicates the importance of possible zoonotic diseases in this region which could affect public health. These results further substantiate the importance of advanced metagenomics analyses to identify neglected tick-borne pathogens which enable researchers to provide efficient mapping roads for the management of emerging and re-emerging infectious diseases.

Keywords: Tick-borne disease; Microbiota; *Ixodes ricinus*; Next-generation sequencing; Emerging pathogens

Introduction

Ticks are worldwide ectoparasites belonging to the Arachnida class, superorder parasitiformes with over 900 species categorized into two families Ixodidae (hard ticks) and Argasidae (soft

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Published Online: June 30, 2023

ticks) (1). Among over 700 hard ticks, *Ixodes ricinus* is one of the most abundant species infesting various hosts, including birds, mammals, and occasionally reptiles which derived from *Acarus* species (2, 3). They meet their nutritional requirements by feeding on the vertebrate's blood hosts and ecological and anthropogenic factors affect *I. ricinus* ticks' population and activity (4). Since *I. ricinus* ticks prefer a warm and humid environment, the temperature and the moisture amount in the air and soil are determinants (5); however, these ticks also occur in areas such as Iceland (6), which has a sub-polar oceanic climate with poor vegetation (7). The availability and the abundance of suitable hosts are other fundamental components. Nevertheless, geographical regions with lowlands, forests, and green pastures are excellent habitats for *I. ricinus* ticks geographical regions with lowlands, forests, and green pastures are excellent habitats for *I. ricinus* ticks (8). Moreover, agriculture-associated activities, biotope alteration, insecticides, and anti-tick vaccination for tick management are considered human-induced elements that impact the frequency of ticks (9). The microbiome (microbiota) is a group of diverse microorganisms present inside and outside another organism (10, 11). They can be symbiotic, commensal, or even pathogenic for the host. The tick microbiome includes different species of bacteria, viruses, and protozoa, some of which are called tick-borne pathogens (12, 13). Ticks carry a variety of microbiota inside their salivary glands, midgut, and ovary, and they can transmit them to their hosts (human or animal) during blood-feeding (14). *Ixodes ricinus* ticks are widely known as vectors of Tick-borne disease (TBDs) pathogens causing medical and veterinary conditions (15). Lyme disease and tick-borne encephalitis (TBE) are caused by *Borrelia burgdorferi* sensu lato and the tick-borne encephalitis virus (TBEV), both harbored by *I. ricinus* (16). Some of the pathogens previously identified to be carried by *I. ricinus* are *Babesia divergens* (17), *Babesia microti*, *Babesia venatorum* (18), *Anaplasma phagocytophilum* (17), *Rickettsia Helvetica* (19), *Rickettsia slovaca*, *Rickettsia monacensis* (20), *Borrelia miyamotoi* (21), *Ehrlichia phagocytophila* (17), Louping ill virus (22), *Bartonella henselae* (23), *Coxiella burnetii* (24), and *Francisella tularensis* (25). Non-pathogenic microorganisms are also present in the tick's microbiome, including those involved in amino acid and lipid metabolism pathways (26). Metagenomics is the science of studying the genetic content of environmental samples. The next-generation sequencing method plays a vital role in analyzing DNA and RNA materials. This high-throughput technology provides a reliable and accurate way of identifying the microorganisms in almost any microbial community (11, 27). The V6 region of the 16S rRNA gene is highly conserved among different bacterial species and can provide reliable high resolution taxonomic classification of bacteria and archaea. This region is also relatively short and easy to amplify and to sequence using PCR-based methods, making it easier and more cost-effective compared to longer regions for large-scale metagenomic studies (27). The present study investigated the bacteria in the midgut of *I. ricinus* ticks collected from the Golestan, Mazandaran, and Gilan provinces in the northern part of Iran. This littoral region of the Caspian Sea is covered by the Hyrcanian forests creating a suitable environment for ticks' activity and reproduction regarding the humidity, temperature, and a vast range of suitable hosts. It is consequential to improve our knowledge about the pathogens in ticks to organize the best and most effective tick management strategies protecting both humans and animals (whether domestic or wild) against TBDs.

Materials and Methods

Study area and population

The study area in the Caspian Sea littoral of Iran included the southern border of the Caspian Sea and the northern heights of Alborz mountains, covering an area of 58,167 Km2 in three provinces of Gilan (37°04′27″N 50°12′ 35″E)

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Mazandaran (36.2262° N, 52.5319° E), and Golestan (37.2898° N, 55.1376° E). The Hyrcanian forest in northern Iran covers 3,400,000 hectares on the Alborz Mountains' northern slopes stretching down to the Caspian Sea coastal areas (Fig. 1). General characteristics of the climate are too much rain in all seasons, especially in autumn and winter, relatively high humidity in all seasons, and low-temperature difference during the day due to moisture.

Sampling

Ticks were collected from domestic animals, i.e., cattle and sheep, in autumn from 6 to 11 Nov. 2021 during the period of tick multiplication. The *I. ricinus* ticks were transferred to the laboratory immediately under ambient, live conditions and identified under stereomicroscope using available taxonomic key for ticks (28). A total number of 39 *I. ricinus* ticks isolated from cattle and sheep were collected in 5 localities (Table 1) (Fig. 1). The collected ticks were from different locations in the coastal area in the north of Iran. Only one male tick was among 39 collected ticks, considered a separate pool. Furthermore, this was from Golestan-Barfatan where no female tick was found. More data, such as location, number of ticks, and hosts, are available in Table 1.

Separation of midgut from *Ixodes ricinus*

To make sure no microorganism contamination from the ticks' surface or the environment enters our study, before doing any operation, the ticks were washed carefully in 70% alcohol for 3 minutes and then rinsed with sterile water alternatively three times. First, the ticks identified in the laboratory were placed individually on a slide, a few drops of molten paraffin were poured on them to keep the ticks fixed on the slide, and the paraffin was allowed to harden. The ticks were dissected under an entomological loop using a small pair of ophthalmic scissors. The ticks were dissected under an entomological loop using a small pair of ophthalmic scissors. The dorsal scutum and the white grape clusters-like salivary glands were completely removed, then the whole intestinal section was separated and transferred to a sterile opener with some distilled water inside and then stored in a -20 °C freezer for DNA extraction steps.

DNA Extraction

DNA of isolated intestines was extracted using a genomic DNA extraction kit (Bio Basic, Canada according to the manufacturer's instructions. Isolates were disrupted by adding 200 mg of glass beads (0.2 mm diameter) Plus 400 µL of lysis buffer (containing 200 M Tris-HCL, pH= 8, 25 mM EDTA, SDS 0.5% W/V, NaCl 250 mM) and vortexed using a Micro smash MS-100R (Tomy Seiko, Japan) at 4000 rpm for the 60 s. Then added 200 µL buffer PP was to microtubes containing the above mixture and incubated at -20 °C for 5 minutes. Finally, DNA was extracted using the chloroform and isopropanol methods (29).

Metagenomics analysis

Genomic DNA was extracted from five pools prepared based on five locations (each pool contained the collected ticks of that location). The final product, a mix of microorganisms' DNA content, was sent to Macrogen Company (S. Korea) for sequencing. The DNA was amplified for V6 region of the 16S rRNA gene by Herculase II Fusion DNA Polymerase kit using 341F forward primer and 850-R reverse primer (primer sequence (CCTACGGGNGG CWGCAG) and (GACTACHVGGGTATCT AATCC), respectively. The library preparation was done by DNA Polymerase Nextera XT kit, which is suitable for working with small genomes. The product was sequenced with a Miseq device, and it was set to sequence the PCR product 300 base pairs from both ends. The MiSeq benchtop sequencer output is over 100,000 reads with a length of 300 and a total of more than 30 million bases. Then analysis was performed on the output Fastq file after checking the quality.

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NGS data analysis

The first step in NGS data analysis is checking the quality of the raw fastq files by Fastqc and MultiQC (30, 31). It is notable that for 16 S amplicon sequencing, having many repeats in the samples is very common. DADA2 software was used to de-noise and join the forward and reverse reads (32). Trimming lengths for forward and reverse reads are very important in this step. Since the primers used were 341F and 850R, the expected length of amplicons is 850−341=509, so with paired-end read length equal to 300, we have 2∗300−509=91 bases overlap without trimming. It is recommended to leave 20 + natural amplicon variance bases for overlap and trim the rest for best results. In checking with Fastqc results, the trim length for forward reads was set at 300 (no trimming) and for reverse reads at 250 (trimming 50 bases from the end). Since the length of the target region (509) is much higher than the read length (300), the risk of having an adapter sequence at the end of forward reads is negligible. Then the resulting OTUs were annotated with the SILVA (33) database assigning each sequence to the nearest species that share at least 90 percent of the sequence. QIIME 2 (34) platform has been utilized for visualization of data.

There are different methods for constructing a phylogenetic tree. The Maximum Likelihood (ML), which is the method we have used in this study infers a phylogenetic tree by calculating the likelihood of observing the given DNA or protein sequences under different evolutionary models and tree topologies. The tree with the highest likelihood is considered the most likely. ML is a powerful method that can incorporate complex models of sequence evolution, but it can be computationally intensive and may require substantial computational resources (35).

Results

Thirty-nine ticks were collected from three neighboring provinces of Gilan, Mazandaran, and Golestan in the north of Iran, creating five pools based on the localities (Fig. 1). Following DNA extraction of each pool and amplification of V6 region of the 16S rRNA gene, sequence reads of 98042 (MOT11311), 114951 (MOT15054), 103101 (MOT15055), 68754 (MOT15056), and 112262 (MOT15061) were obtained from sequencing by Miseq Illumina. A large number of bacteria and the archaea phylum Woesearchaeota were observed in the tick microbiome community data. Because the total read count of Woesearchaeota was only 14, so we considered it low confidence and removed it from our data focusing only on bacteria. Also, there were 275 unassigned reads. The dominant taxonomy levels of phylum, class, order, family, genus, and species belonged to *Proteobacteria*, *Alphaproteobacteria*, *Rickettsiales*, *Rickettsiales Incertae sedis*, *Candidatus midichloria*, and *Burkholderia* sp. A3 (2016), while the minimum belonged to *Fusobacteria*, *Holophagae*, *Holophagae.* Subgroup 7, Nocardiaceae, *Curtobacterium*, and *Curtobacterium* sp. SW30, respectively.

Fourteen phyla were found in the five pools together (Figs. 2 and 3). Phylogenetic relationship of microbiota from *I. ricinus* intestine prepared by metagenomics analyses is shown in Fig.

Following Proteobacteria with over 93% frequency, Bacteroidetes, Actinobacteria, and Firmicutes were the most frequent phyla consisting of over 1% of the reads, and they exist in all pools despite low abundance in some. The rest (6% of the reads) were the reads with no related bacteria identified or bacteria with low read counts that were removed.

The analysis showed a difference in the relative abundance of the pool appointed to the male tick with the female's pool. The Armatimonadetes, Ignavibacteriae, Fusobacteria, and Saccharibacteria phyla were seen in the male tick microbiota but not in females. In contrast, Deinococcus-Thermus and Spirochaetae were only observed in females. Furthermore, the dominant phyla were different in the male sample. For example, the Actinobacteria phylum is far more frequent in the only male tick analyzed.

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Published Online: June 30, 2023

Fig. 3 shows the relative abundance of each sample in 4-6 of taxonomy levels (level 4: order, level 5: family, level 6: genus). The abundance of the top 30 detected bacteria with the most frequencies can be found in Table 2. Our results showed that pathogenic bacteria in the Rickettsia genera are the predominant pathogenic organisms in our collected ticks which could be representative of the higher presence of rickettsiosis in the region of study. Indeed, the presence of *Burkholderia*, *Corynebacterium*, *Coxiella* and *Pseudomonas* in our samples showed the probable importance of neglected diseases but no significant difference was observed between sampling locations.

Table 1. Detailed information of tick sampling in the north of Iran

Sample ID	Province (Village)	Location	Number of tick(s)	Gender	Host
MOT1131	Golestan-Barfatan	36°53′05″N 54°47′54″E	1	Male	Sheep
MOT15054	Mazandaran (Klardasht-Banafsheh Deh)	36°30′00″N 51°15′00″E	11	Female	Cattle
MOT15061	Mazandaran (Chamestan-Joorband)	36°26′26″N 52°07′16″E	8	Female	Sheep
MOT15055	Mazandaran (Salman Shahr-Sisara)	36°42′12″N 51°13′50″E	2	Female	Cattle
MOT15056	Gilan (Narenjbon)	37°04′27″N 50°12′35″E	17	Female	Cattle

Table 2. Metagenomics data of relative abundance of top 30 identified bacteria at genus and species level in five pooled samples of *Ixodes ricinus* based on genus and species

Bacteria	RA *	Bacteria	RA*
Candidatus midichloria	0.69380803	*Burkholderia* sp. A3(2016)	0.058673062
Rickettsia	0.093340712	*Ralstonia* sp. 14I	0.012101088
Ralstonia	0.058673062	*alpha proteobacterium* Gsoil 1161	0.005054418
Sediminibacterium	0.012101088	*Bradyrhizobium elkanii*	0.003320421
Bradyrhizobium	0.008091988	*Bradyrhizobium* sp. CCBAU 51357	0.001586423
Nitrobacter	0.006222714	*Staphylococcus chromogenes*	0.001463445
Aquabacterium	0.006161225	*Pseudomonas putida*	0.001180594
uncultured	0.00538646	*Wautersiella* sp. MBG55	0.001119105
uncultured	0.004968333	*Pseudomonas putida* W15Oct28	0.000836254
Mesorhizobium	0.004722376	*uncultured Deinococci bacterium*	0.000823956
Burkholderia-Paraburkholderia	0.004710078	*Lactobacillus delbrueckii* subsp. *delbrueckii*	0.000737871
Reyranella	0.004316547	*Zea luxurians*	0.000737871
Acinetobacter	0.002803911	*uncultured Streptococcus* sp.	0.000725573
Shewanella	0.002607145	*Lactococcus lactis*	0.000700978
Pseudomonas	0.00252106	*wastewater metagenome*	0.000676382
Corynebacterium1	0.002299699	*Pseudomonas* sp. HOT12	0.000676382
Staphylococcus	0.002152124	*bacterium#1 from Psoroptes ovis*	0.000627191
Sulfuritalea	0.002102933	*Paracoccus* sp. B-1012	0.000565701
Sphingobacterium	0.001807785	*uncultured Methylocella* sp.	0.000553403
uncultured	0.001733997	*Shewanella* sp. AK55	0.000541106
Citrobacter	0.001623317	*Staphylococcus* sp. NR 4-12	0.00051651
Coxiella	0.001611019	*Sphingobacterium multivorum*	0.00051651
Enhydrobacter	0.001537232	*Stenotrophomonas maltophilia*	0.000430425
Rhizomicrobium	0.001475742	*Sediminibacterium* sp. OTC15	0.000405829
Streptococcus	0.001426551	*Nevskia terrae*	0.000405829
Nevskia	0.001401955	*Staphylococcus* sp. J33	0.000393531
Bacillus	0.001266679	*Caulobacter* sp. UR 6-06	0.000356638

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Table 2. Continued ...

Delftia	0.001266679	*Comamonas aquatica*	0.00034434
Empedobacter	0.001168296	*Bacterium* R78	0.00034434
Sorangium	0.001155998	*Microbacterium laevaniformans*	0.000319744

*Relative Abundance

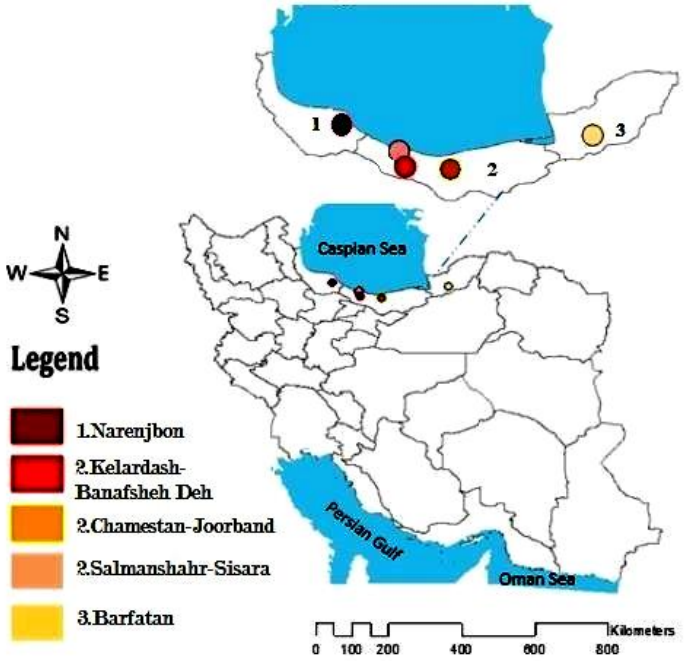

Fig. 1. Geographic distribution of *Ixodes ricinus* sampels in Gilan (No. 1), Mazandaran (No. 2) and Golestan (No. 3) provinces in the north of Iran using ArcGIS software. Esri

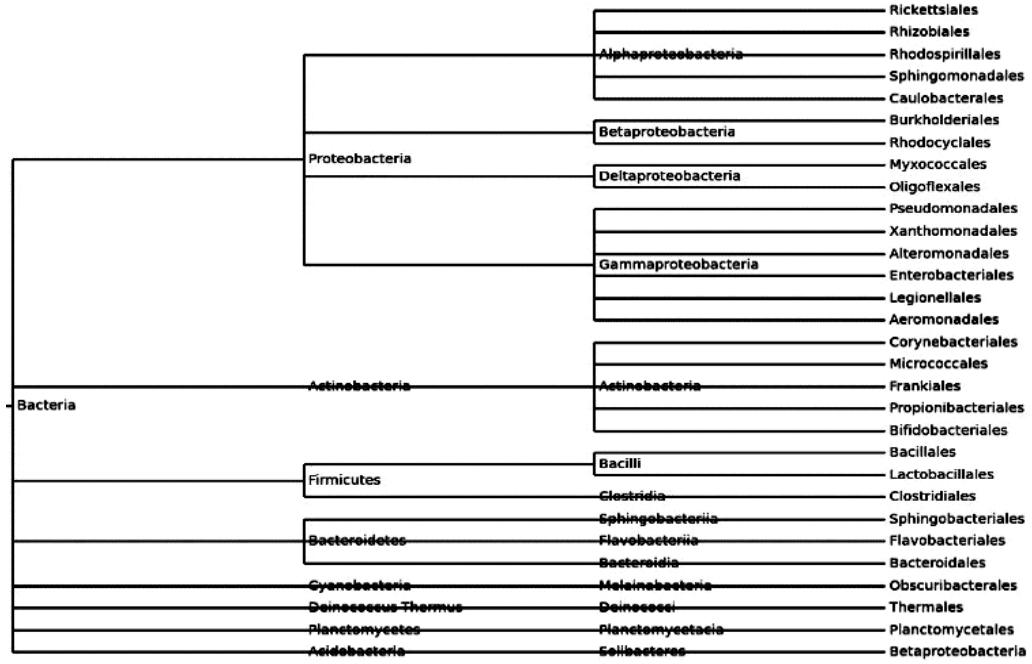

Fig. 2. Phylogenetic relationship of microbiota from *I. ricinus* intestine prepared by metagenomics analyses in 4 taxonomy levels of domain, phylum, class, and order using Biopython 1.81, Phylo library

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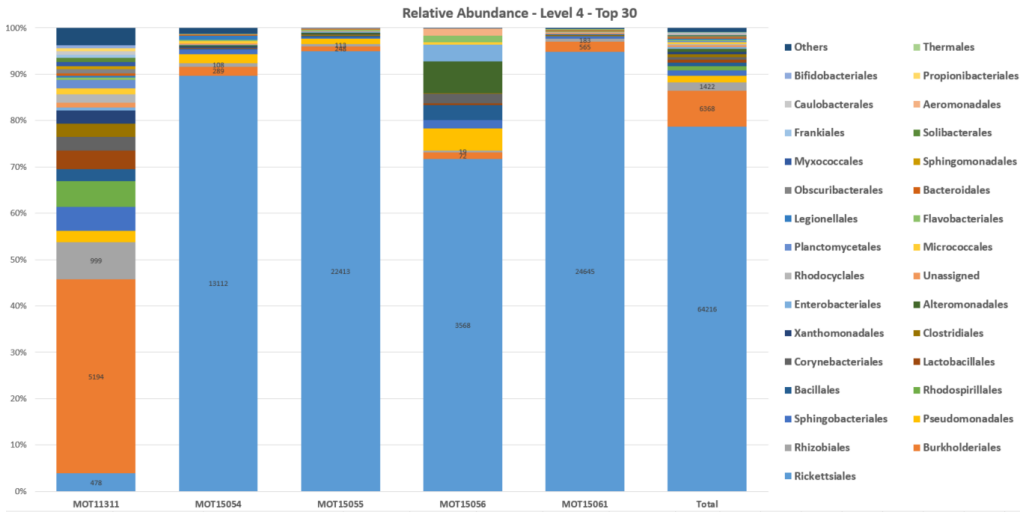
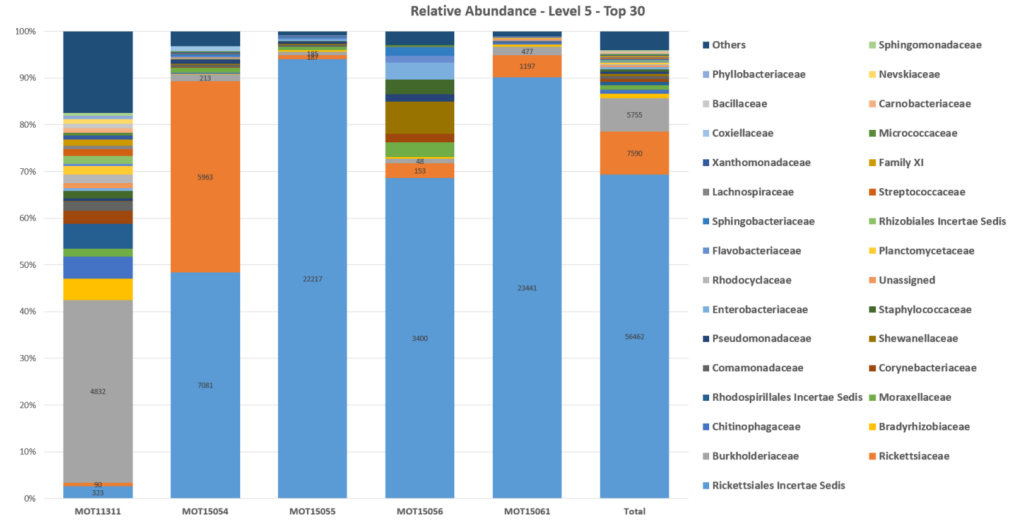
A



B



C

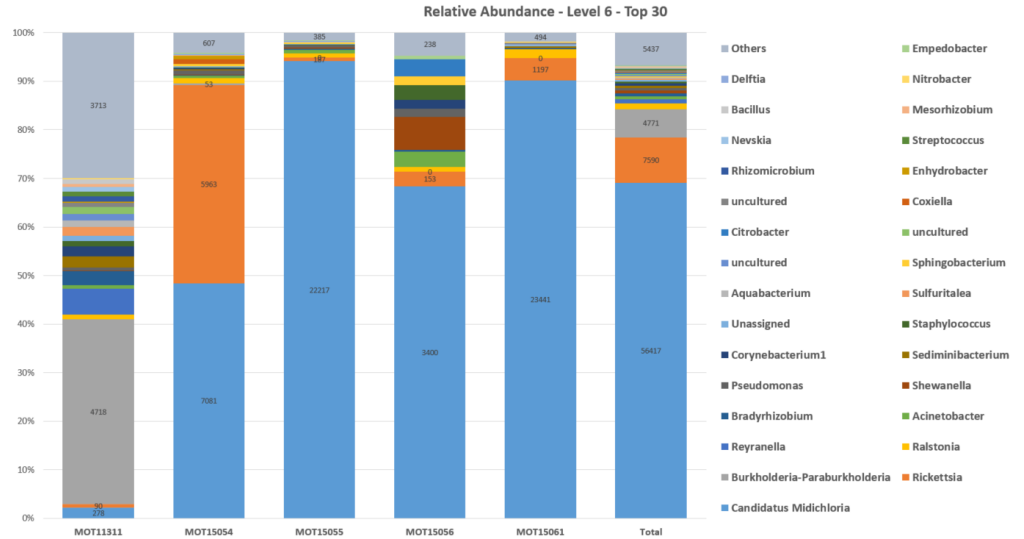


Fig. 3. Metagenomics data of relative abundance of top 30 bacteria found in five sample pools of *Ixodes ricinus* (A= order, B= family, C= genus) using Matplotlib 3.7.1 library

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Published Online: June 30, 2023

Discussion

This study was conducted to reveal and identification of midgut microbiota of *I. ricinus* ticks and their impact on disease distribution in 3 Northern provinces of Iran. Our data exhibited 14 phyla, of which Bacteria, Proteobacteria, Actinobacteria, and Firmicutes were the most abundant. In our study, Proteobacteria were the most abundant, which was consistent with a study in Italy and Spain (36, 37). In a study conducted in Iran on *Hyalomma anatolicum* tick, Proteobacteria was also the most abundant (38).

The present study also investigated the relative abundance of bacterial species in the *I. ricinus* tick. Among the different bacteria examined, 30 were more abundant in 5 pools of ticks. Similar to the present results, Bacteria, Actinobacteria, and Firmicutes were the most abundant after Proteobacteria, which accounted for more than 0.01% of the readings (39). Interestingly, in the study in Italy and Slovakia (36, 40), Actinobacteria were collected as one of the dominant groups in *I. ricinus* ticks and were much more abundant than the results of our research. According to the findings of previous studies, genus of *Candidatus midichloria* (0.69), *Rickettsia* (0.09), *Ralstonia* (0.05), and species of *Burkholderia* sp. (0.05), *Ralstonia* sp. 14I (0.01), alpha-proteobacterium Gsoil 1161 (0.00) was found with the highest frequency.

The results of our study were consistent with those of Spain (37) that *Candidatus midichloria* the most abundant bacteria in *I. ricinus* ticks. The high abundance of this bacterium may indicate the endosymbiosis of *Candidatus midichloria* mitochondrii (CMM)(41), whose high abundance in *I. ricinus* material is associated with its unique ability to localize inside mitochondria in the tick ovary. Because this endosymbiont is consistently detected in oocytes, CMM is thought to be maternally inherited (42). The results of the present study were consistent with the previous studies, so that in Italy and Slovakia, the Rickettsia genus was associated with large amounts in the collected *I. ricinus* ticks (36, 40). Also, another study identified a close relationship between some tick symbionts and the potential presence of pathogenic Rickettsia or a parasitoid in ticks (43). In a study in Iran, Rickettsia was observed in the intestine of *Hyalomma anatolicum* tick (38). Another pathogen observed in the results of our study was *Coxiella*, which was not found in previous studies (39). This pathogen is the cause of Q fever, which is one of the common diseases between humans and animals. This disease affects many populations including human and animals every year and carries a high disease burden (44). *Coxiella* (45), *Rickettsia* (46), flavivirus (47), Crimean-Congo heamorraghic fever (48), and *Burkholderia* (49) are among major health challenges impacting human and animals in Iran. As these diseases are zoonoses, they cause a significant disease burden for health systems every year (50).

Also, our data showed sex-dependent differences in microbiota in ticks. Unfortunately, due to financial constraints, we could not collect more ticks and only one male tick was observed in our study. The only male tick analyzed showed significantly higher bacterial richness and greater overall diversity than its females. A less diverse microbiota in female ticks has also been previously reported for some other tick species and in previous studies, the sample size of ticks was similar to our study (51, 52). However, in a study in America in 2021, female microbiota showed a significantly higher bacterial richness in one of the areas. Still, in general, it had less diversity than its male counterparts (53). The current study can provide preliminary data for further investigations. We also used the new metagenomics method to study and identify the tick's midgut, which showed a significant difference in the different types of pathogenic bacteria. Finally, this study observed zoonotic pathogens in the tick’s intestine, indicating the importance of zoonotic diseases in the region.

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Published Online: June 30, 2023

Conclusion

In this study, the frequency of pathogenic bacteria in ticks was higher in males than that of females. Metagenomics enabled us to identify bacteria such as *Coxiella*, *Rickettsia* and *Burkholderia* which cannot be easily recognized by routine methods. The presence of such pathogenic bacteria indicates the importance of possible zoonotic diseases in this region which could affect the public health meaningfully. Overall, these results further substantiate the importance of advanced metagenomics analyses to identify neglected tick-borne diseases which provide mapping roads for management of emerging and re-emerging infectious diseases.

Acknowledgements

This work is supported by the Pasteur Institute of Iran (Code TP-9353). The authors would like to thank the Pasteur Institute of Iran for their cooperation during the study. Due to the importance of the study, the participants in this research were included in the list of authors.

Ethical considerations

There was no human and animal study that needed ethical concerns.

Conflict of interest statement

The authors declare there is no conflict of interests.

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