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Nonspecific Bacterial Flora Isolated from the Body Surface and Inside *Ixodes ricinus* Ticks

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Abstract

Ixodes ricinus and other representatives of the order Ixodida are vectors of typical pathogens: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilium*, *Babesia* spp., a tick-borne encephalitis virus, and other microorganisms which are important from a medical and veterinary point of view. The presented study focuses on the verification of nonspecific bacterial flora of *I. ricinus*. We analyzed ticks collected in a forest region in Silesia, an industrial district in Poland. Methods of classical microbiology and biochemical assays (API 20 NE test, API Staph test and MICRONAUT System) were used for isolation and identification of microorganisms living on the body surface of *I. ricinus* and inside ticks. The results show the presence of various bacteria on the surface and inside ticks' bodies. During the study, we isolated *Acinetobacter lwoffi*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Achromobacter denitrificans*, *Alcaligenes faecalis*, *Stenotrophomonas maltophilia*, *Pseudomonas oryzihabitans*, *Micrococcus* spp., *Kocuria varians*, *Staphylococcus lentus*, *Kocuria kristinae*, *Streptococcus pneumoniae*, *Rhizobium radiobacter*, *Staphylococcus xylosus*. Majority of the isolated species are non-pathogenic environmental microorganisms, but some of the isolated bacterial strains could cause severe infections.

Key words: *Ixodes ricinus*, microbiological diagnostic, nonspecific bacterial flora

Introduction

Ixodes ricinus, as well as other representatives of the order Ixodida, belongs to mites of great biomedical significance. They are parasites of vertebrates and stay in a humid environment during external nonparasitic periods. Ticks are a potential source of viral, bacterial, and protozoan infections of the hosts. Co-evolution of ticks and microorganisms is the cause of the close, specific and strong connection between vector and the host (Sonenshine, 1993). Because the specificity of the vector-microorganism interaction is so strong, we generally use the term "tick-borne diseases" (TBD) to describe specific diseases transmitted by certain ticks. Typical pathogens transmitted by *I. ricinus* are *Borrelia burgdorferi* sensu lato (Stańczak *et al.*, 2000; Paulauskas *et al.*, 2008), *Anaplasma phagocytophilum* (Stańczak *et al.*, 2004), *Babesia* spp. (Słodki *et al.*, 2011), and a tick-borne encephalitis virus (Makówka *et al.*, 2009). This does not mean that other microorganisms are excluded, bacterial flora which colonises these mites is abundant and diverse (Stojek *et al.*, 2005; Murrell *et al.*, 2003). From a medical and veterinary point of view, the ability of ticks to carry other non-specific pathogens that may cause additional symptoms occurring after a tick bite, should prompt the analysis of the whole non-specific flora.

The aim of this study was to identify non-specific bacteria transmitted by *I. ricinus*.

Experimental

Materials and Methods

I. ricinus ticks, were collected during a spring-summer peak activity of this species in a forest region in Silesia, an industrial district in Poland. Thirty-six adult individuals were collected, among them 75% were females and 25% males.

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Bacteriological diagnostics. Bacterial flora living on the surface and inside their bodies was isolated from the collected ticks. In order to isolate bacteria from the tick's surface, the ticks were washed in sterile physiological saline solution. Then 100 µl of the solution was transferred to nutrient agar (BTL, Łódź, Poland) to multiply bacteria which were later plated on blood agar plates. The plates were incubated for 48 h at 37°C in aerobic conditions.

To isolate bacteria from inside of the ticks, ticks were washed first in 1 ml of 96% ethanol to kill bacteria present on the surface. Then the ticks were transferred to physiological saline solution and were mechanically disrupted. Then 100 µl of the solution was transferred to nutrient agar (BTL, Łódź, Poland) to multiply bacteria which were later plated on blood agar plates. The plates were incubated for 48 h at 37°C in aerobic conditions.

After 48-hours of incubation on blood agar plates, the morphological properties of bacterial colonies were assayed and the following characteristics were recorded: shape, size, surface structure, protruding above the surface, colour, transparency, texture, type of hemolysis. Based on morphological differences of colonies, we isolated pure cultures.

We assayed Gram type and if Gram-negative rods were observed on stained microscopic slide, we plated bacteria on selective media: Mac Conkey Agar (BTL, Łódź, Poland), Cetrimide Agar (BTL, Łódź, Poland), King B Agar (BTL, Łódź, Poland). In case of Gram-positive cocci we tested for the presence of catalase and the bacteria were plated on the Chapman Agar (BTL, Łódź, Poland). All cultures were incubated for 24 hours at 37°C in aerobic conditions.

Biochemical studies. To identify species isolated from ticks, the API 20 NE and API Staph tests (BioMérieux, Marcy l'Etoile, France) were used according to the instructions supplied by the manufacturer. The results were decoded using the software "apiweb" available on the website of the manufacturer (https://apiweb.biomerieux.com).

Bacterial strains identified neither by classical microbiology methods nor using the API 20 NE and API Staph were analysed by a semi-automatic MICRONAUT System for identification and resistance testing of bacteria and yeasts (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany). The material for analyses was prepared according to the manufacturer's insrtuctions.

Results

Among thirty-six tested ticks, mesophilic microorganisms were found only in 30 samples. In six bacterial samples, bacterial flora was detected either on the surface or inside of the tick. Microorganisms were more frequently isolated from the body surface of the ticks. The bacteria living on the surface of the ticks' bodies are largely aerobic microorganisms or facultative aerobes. The flora living inside of the body consisted mainly of anaerobes and facultative anaerobic microorganisms, and aerobic microorganisms occurred rarely.

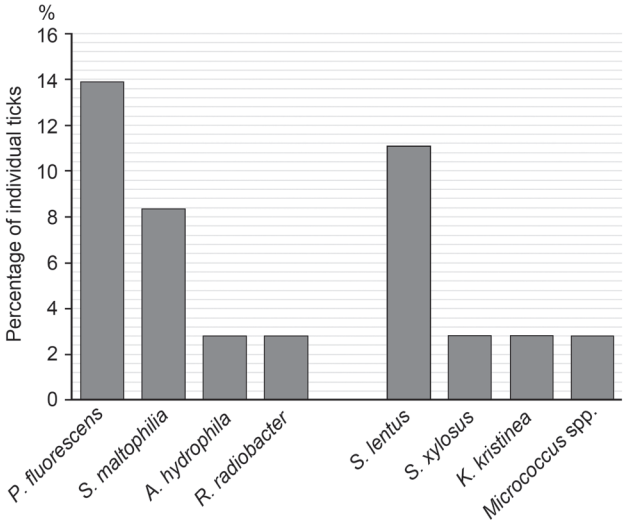

Fig. 1. Bacterial flora on the body surface of *I. ricinus*

Classical microbiology methods allowed detection of ten strains of Gram-negative rods and twenty-nine strains of Gram-positive cocci living on the surface of the analysed ticks. Using classical methods, we were able to identify few species present on the surface, *Pseudomonas fluorescens* (14%, Fig. 1.), *Aeromonas hydrophila, Rhizobium radiobacter* occurred most frequently among the Gram-negative rods, *Stenotrophomonas maltophilia* isolates were identified using automated MICRONAUT system.

Among the cocci, the following species were detected: *Staphylococcus lentus* (11%, Fig. 1), *Staphylococcus xylosus*, *Kocuria kristinea*, and *Micrococcus* sp.

From the inside of tics we isolated *P. fluorescens*, *A. hydrophila*, *Achromobacter denitrificans*, *Alcaligenes faecalis*, *S. maltophilia*, *Acinetobacter lwoffii* and *Pseudomonas oryzihabitans*.

Among the Gram-positive cocci living inside the ticks, there were detected the same species of microorganisms as on the body surface: *S. lentus*, *S. xylosus*, *K. kristinea*, *Kocuria varians* and the cocci of the genus *Micrococcus*, and *Streptococcus pneumoniae.*

The most commonly identified rods living inside the ticks were: *A. lwoffii* (11%), *P. fluorescens* (8%) and *S. maltophilia* (8%), while among the cocci *S. lentus* (11%) species were the most frequent (Fig. 2).

The identification of isolated bacterial strains showed that the greatest diversity of bacterial flora was present on the surface and inside the bodies of female ticks. Eight species of Gram-negative rods and five species of Gram-positive cocci were isolated from female ticks

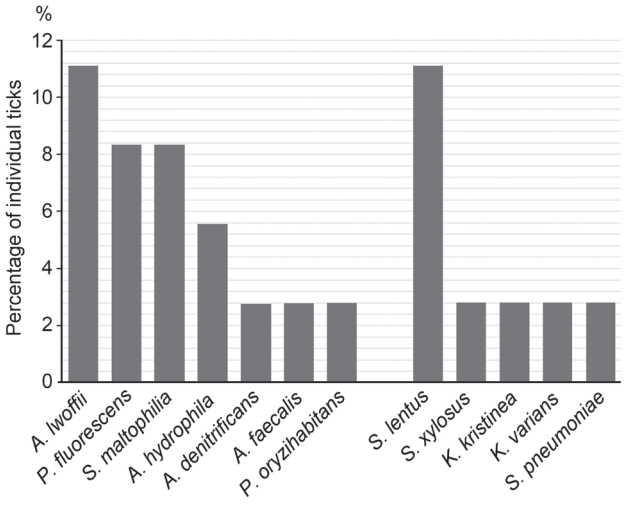

Fig. 2. Bacterial flora inside *I. ricinus*

(Table I). In male ticks, we observed much lower diversity of bacterial flora on the surface and inside their bodies. Two species of Gram-negative rods and two species of Gram-positive cocci were identified (Table I).

Discussion

A lot has been published about health problems associated with the transfer of typical tick-related pathogens. However, researchers pay less attention to microorganisms which are nonspecific for ticks. It can be assumed that bacteria transmitted by ticks are mainly nonpathogenic environmental microbes. Nevertheless, the representatives of *I. ricinus* species have contact with different hosts, thus analysing potential transmission of various microorganisms by these mites is very important.

Earlier publications about the colonisation of *I. ricinus* by microorganisms identified the presence of various bacteria on the body surface of the ticks, including *Pasteurella pneumotropica/haemolytica*, *Pantoea agglomerans*, *Serratia marcescens*, *Serratia plymuthica*, *Aeromonas hydrophila*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Stojek and Dutkiewicz, 2004). Furthermore, the possibility of transmission of *Salmonella enteritidis, Listeria monocytogenes, Erysipelothrix rhusiopathiae* by *I. ricinus* was described (Siuda, 1991). Rahman and Rahman (1980) discovered the presence of *Escherichia*, *Proteus*, *Pseudomonas* and *Staphylococcus* species in *Rhipicephalus sanguineus* and *Haemaphysalis leachi leachi* ticks. In these species of ticks, horizontal transmission of *Staphylococcus aureus* and *Staphylococcus epidermidis* was also described (Adejinmi and Ayinmode**,** 2008.). Studies on the presence of Gram-negative bacteria in hard and soft ticks, *Hyalomma dromedarii*, *Serratia liquefaciens*, *Stenotrophomonas maltophilia*, *Klebsiella ornithinolytica* and *Aeromonas hydrophila* were identified. Five species were isolated from *Argas persicus*: *Rahnella aquatilis*, *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Chryseomonas luteola* and *Chryseobacterium meningosepticum* (Montasser, 2005). Also fungi were identified as important elements of the flora on the surface and inside ticks; *Scopulariopsis brevicaulis* was isolated from *Dermacentor variabilis* ticks (Yoder *et al*., 2003), *Penicillium spp*, *Fusarium spp*, and *S. brevicaulis* were found on the body surface of *I. ricinus*, *D. reticulatus* and *D. marginatus* (Samsinakova *et al*., 1974).

Published research suggests differences between the microflora associated with different species of ticks which live in various environments (Murrell *et al*., 2003; Martin and Schmidtmann, 1998; Amoo *et al*., 1987). However, it does not mean that isolated microorganisms are potentially pathogenic and may influence a clinical presentation after a tick bite or tick contact with injured skin. It is more noticeable that some species of bacteria are repeatedly observed in ticks. Comparison of literature data with our results shows that *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia* and *Aeromonas hydrophyte* are isolated from ticks in different regions of the world. Previously, the occurrence of bacteria such as: *S. lentus*, *S. xylosus R. radiobacter*, *K. kristinae*, *A. denitrificans*, *A. faecalis*, *P. oryzihabitans*, *K. varians*, *S. pneumoniae*, *A. lwoffii* in the *I. ricinus* ticks was not observed.

In our studies, *S. lentus*, *A. lwoffii, S. maltophilia* and *P. fluorescens* were isolated most frequently, and association of these species with ticks may have epidemiological and medical consequences. Generally, *S. lentus* is

Table I
Bacterial flora of male and female *I. ricinus* ticks

Sex of *I. ricinus* ticks	Area of the ticks' body	Rods	Cocci
Males	surface	*P. fluorescens*	*S. lentus*, *S. xylosus*
	inside	*S. maltophilia*	*S. lentus*
Females	surface	*P. fluorescens, A. hydrophila*, *R. radiobacter*, *S. maltophilia*	*Micrococcus* spp., *S. lentus*, *K. kristinae*
	inside	*A. lwoffii*, *P. fluorescens, A. hydrophila, A. denitrificans, A. faecalis*, *S. maltophilia*, *P. oryzihabitans*	*Micrococcus* spp., *K. varians*, *S. lentus, K. kristinae*, *S. pneumoniae*

described as a non-pathogenic species. However, it is sometimes isolated from patients with urinary tract infections (Stepanović *et al*., 2003; Stepanović *et al*., 2005). Moreover, Nawrotek *et al.* (2010) isolated coagulase-negative staphylococci (CNS) such as *S. lentus* and *S. xylosus* from the milk of cows with symptoms of the mammary gland inflammation (*mastitis*). There are genes encoding enterotoxins in the genome of these staphylococci, and as a consequence, *S. lentus* and *S. xylosus* can potentially cause enterotoxin poisoning in people who have consumed milk and dairy products. At the same time, the occurrence of these staphylococci in ticks creates the opportunity of spreading virulent bacteria that produce toxins among cattle.

Acinetobacter lwoffii and *S. maltophilia* may have clinical relevance in the case of infections in patients with lower immunity. *A. lwoffii* are Gram-negative rods constituting the physiological flora of human skin. However, these bacteria are dangerous for hospitalised patients (Ku *et al*., 2000). These rods show tropism toward the mucous membrane of urinary tract and often are multidrug resistant (Tega *et al*., 2007). *A. lwoffii* seems to be more pathogenic for animals, *e.g.* birds, causing severe respiratory diseases (Robino *et al*., 2005). There was also a case of fulminant community-acquired pneumonia in humans, probably caused by *A. lwoffii* with fatal outcomes (Toyoshima *et al*., 2010). Such infections show that the pathogenesis and transmission of *A. lwoffii* requires further study, and the presence of these bacteria in ticks infesting animals and humans cannot be ignored. *S. maltophilia* and *Acinetobacter* spp. are widespread in environment. Their medical significance is mainly limited to the hospital environment, because these rods cause nosocomial infections such as injured skin infections and soft tissue infections, pneumonia, meningitis, endocarditis and urinary tract infections. (Araoka *et al.*, 2010). Patients with cystic fibrosis show an increased risk of infection by *S. maltophilia* (Colin and Rabin, 2011). Although the frequency of *S. maltophilia* infections is not high, these cases pose serious problems because the risk of death arising from these infections is estimated at 20–70% (Farrell *et al*., 2010). Moreover, *S. maltophilia* rods have natural resistance mechanisms and therefore they may have greater medical significance. These rods are not only responsible for the pathogenesis of diseases difficult to treat, but they are a source of drug resistance genes for other bacteria. Researches described the possibility to transfer resistance genes from strains of *S. maltophilia* to other non-fermenting rods such as *P. aeruginosa* and the other *Enterobacteriaceae* (Izydorczak and Stefańska, 2007). Thus, every way of spreading of *S. maltophilia*, including infection by ticks, may have clinical relevance, especially in the case of co-infection with typical ticks pathogens.

Non-fermenting rods *P. fluorescens* constituted a quite large percentage of isolated bacteria in our studies. However, they have no clinical significance outside the hospital environment. They are isolated very rarely in immunocompromised patients. It is believed that some strains of *P. fluorescens* may be of great importance for intestinal inflammation (Madi *et al.*, 2010).

The number of other isolated bacteria did not exceed 3%. Some are potentially pathogenic species such as *S. pneumoniae* and *A. hydrophila* (Jacobs, 2007; Saidi *et al*., 2011). However, the majority of them are mostly opportunistic pathogens that infect immunocompromised patients. Group of opportunistic pathogens isolated in our study consists of *S. xylosus*, *R. radiobacter, K. kristinae*, *A. faecalis*, *P. oryzihabitans*, *A. denitrificans* (Orrett and Shurland, 1998; Sood *et al*., 2010; Martinaud *et al.*, 2008; Freney *et al.*, 1988; Mensah *et al.*, 1989).

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