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STUDIES ON THE OCCURRENCE OF GRAM-NEGATIVE BACTERIA IN TICKS: *IXODES RICINUS* AS A POTENTIAL VECTOR OF *PASTEURELLA*

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Abstract: A total of 372 *Ixodes ricinus* ticks (101 females, 122 males, and 149 nymphs) collected by flagging in 6 mixed woodlands of eastern Poland were examined by culture for the presence of internal Gram-negative bacteria other than *Borrelia burgdorferi.* Adult ticks were examined in pools of 2 specimens each and nymphs were examined in pools of 3-5 specimens each. Ticks were disinfected in 70% ethanol and homogenized in 0.85% NaCl. The diluted homogenate was inoculated onto 3 kinds of agar media: buffered charcoal yeast extract (BCYE-α) for isolation of fastidious Gram-negative bacteria, eosin methylene blue agar (EMB) for isolation of enterobacteria, and tryptic soya agar for isolation of all other non-fastidious Gram-negative bacteria. The Gram-negative isolates were identified with the API Systems 20E and NE microtests. A total of 9 species of Gram-negative bacteria were identified, of which the commonest were strains determined as *Pasteurella pneumotropica*/*haemolytica*, which were isolated on BCYE-α agar from ticks collected in all 6 examined woodlands. The total number of these strains (49) exceeded the total number of all other strains of Gram-negative bacteria recovered from ticks (30). Of the total number of examined ticks, the minimum infection rate with *Pasteurella pneumotropica*/*haemolytica* was highest in females (18.8%), and slightly lower in males (12.3%) and nymphs (10%). Besides *Pasteurella pneumotropica*/*haemolytica*, the following species of Gram-negative bacteria were isolated from examined ticks: *Pantoea agglomerans*, *Serratia marcescens*, *Serratia plymuthica* on EMB agar and *Aeromonas hydrophila*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* on tryptic soya agar. Minimal infection rates with these bacteria were low, ranging from 0.7-5.9%. Of the isolated bacteria, *Chromobacterium violaceum*, *Pasteurella pneumotropica*/*haemolytica*, *Pseudomonas aeruginosa*, and *Serratia marcescens* are potentially pathogenic for man and/or animals. In particular, the common occurrence of *Pasteurella pneumotropica*/*haemolytica* in *Ixodes ricinus* ticks poses a potential risk of pasteurellosis for humans and animals exposed to tick bites.

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INTRODUCTION

Ixodes ricinus L., inhabiting deciduous and mixed forests and surrounding areas, is the most common tick in Europe, including Poland [19]. It represents a significant health hazard for humans and domestic animals as a vector of multiple pathogens, of which the most important are: *Flavivirus* causing tick-borne encephalitis, spirochete *Borrelia burgdorferi* causing Lyme disease, rickettsia *Anaplasma phagocytophilum* causing ehrlichiosis (ana-

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plasmosis), Gram-negative bacterium *Francisella tularensis* causing tularemia, and protozoans *Babesia* spp. causing babesiosis in animals, rarely in humans [4, 8, 15, 16, 17, 20]. *Ixodes ricinus* has also been reported as participating in the transmission of many other pathogenic micro-organisms, including: virus of Louping ill, virus of Crimean-Congo hemorrhagic fever, viruses of the groups Uukuniemi and Kemerovo, *Rickettsia slovaca*, *Coxiella burnetii*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Erysipelothrix rhusiopathiae*, *Brucella melitensis*, *Theileria mutans*, *Theileria sergenti* [18]. Most probably this list is not yet complete, as recently we have detected DNA of *Toxoplasma gondii* in *Ixodes ricinus*, thus demonstrating the possibility of transmission of toxoplasmosis by these ticks [21].

The aim of the present work was to examine the role of *Ixodes ricinus* as a potential vector of Gram-negative bacteria pathogenic for humans and animals (except for *Borrelia burgdorferi* which will be a subject of a separate work) by recovery of these organisms from ticks collected in eastern Poland on 3 kinds of bacteriological media.

The preliminary results of this work have been presented elsewhere [22].

MATERIALS AND METHODS

Collection of ticks. The infection of ticks with Gram-negative bacteria was studied in 6 localities (woodlands) of the Lublin province (eastern Poland) (Fig. 1). Ticks were collected by dragging a light woolen flag (60 × 90 cm) over the lower vegetation, mostly on forest paths, in glades, and at the edge of the woods.

All examined woodlands were mixed forests, composed mainly of English oak (*Quercus ruber*), Scots pine (*Pinus sylvestris*), European alder (*Alnus glutinosa*), European hornbeam (*Carpinus betulus*), white warty birch (*Betula verrucosa*), and European beech (*Fagus sylvatica*).

Ticks were collected during May 2003 and May 2004 in the following localities: 1) Dąbrowa – inside a suburban forest south of the city of Lublin, on forest paths; 2) Kozłówka – on the territory of a recreational woodland, in glades and on forest paths; 3) Rogóźno – on the territory of a lakeland northeast of Lublin, on the area surrounding the forest; 4) Krasne – on the territory of a lakeland in the vicinity of Rogóźno, in the area surrounding the forest; 5) Zwierzyniec – on the territory of the Roztoczański National Park, in glades and on forest paths; 6) Sobibór – in a woodland near the boundary of the country, on the area surrounding the forest. Collected ticks were placed in test tubes with a few blades of grass and preserved at room temperature until investigation.

Isolation of bacteria from ticks. Adult ticks (females and males) were examined for the presence of internal Gram-negative bacteria in pools of 2 specimens each and nymphs were examined in pools of 3-5 specimens each. Ticks were washed in saline (0.85% NaCl), submerged for 3 min in 70% ethanol for disinfection of external surface, washed twice in saline, and finally triturated in 0.5 ml of saline with sterile glass homogenizer. The diluted homogenate was inoculated by spreading 0.1 ml aliquots on the agar surface with a sterile glass spatula, onto the following media:

- Buffered charcoal yeast extract (BCYE-α, Oxoid) for isolation of fastidious Gram-negative bacteria.
- Eosin methylene blue agar (EMB agar, Difco) for isolation of enterobacteria (*Enterobacteriaceae*).
- Tryptic soya agar (Difco) for isolation of all other non-fastidious Gram-negative bacteria.

Figure 1. Map showing area of study: Lublin province in eastern Poland.

The buffered charcoal yeast extract (BCYE-α) agar is used mostly for isolation of *Legionella* [3, 23], but could be useful also for isolation of *Francisella tularensis* and related fastidious bacteria [23]. In this work, the charcoal yeast extract (CYE) agar base was used supplemented with the Growth Supplement SR 110 A (ACES buffer/potassium hydroxide, ferric pyrophosphate, L-cysteine HCl, α-ketoglutarate) (Oxoid, England).

Inoculated BCYE-α, EMB and tryptic soya plates were incubated for 24-48 hrs at 37ºC. Grown colonies were Gram-stained and Gram-negative strains were isolated on tryptic soya agar slopes.

The Gram-negative isolates were identified with the use of the API Systems 20E and NE microtests (bioMérieux, Marcy l'Etoile, France).

RESULTS

The results of the study are presented in Table 1. Altogether, 9 species were identified, of which 1 (*Pasteurella pneumotropica*/*haemolytica*) was isolated on BCYE-α agar, 3 (*Pantoea agglomerans*, *Serratia marcescens*, *Serratia plymuthica*) on EMB agar, and 5 (*Aeromonas hydrophila*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*) on tryptic soya agar (TSA). The species

Table 1. Gram-negative bacteria isolated from *Ixodes ricinus* ticks collected in various woodlands on the territory of Lublin province, eastern Poland.

Woodland	Dąbrowa			Kozłówka			Rogóźno			Krasne			Zwierzyniec			Sobibór			Total		
Stage	♀♀	♂♂	NN	♀♀	♂♂	NN	♀♀	♂♂	NN	♀♀	♂♂	NN	♀♀	♂♂	NN	♀♀	♂♂	NN	♀♀	♂♂	NN
No. of examined ticks	10	5	13	8	10	15	16	22	10	11	11	5	36	34	73	20	40	33	101	122	149
BCYE-a agar																					
Pasteurella pneumotropica/ haemolytica complex	2 *20*	1 *20*	1 *7.7*	2 *25*	2 *20*	0	0	6 *27.3*	0	2 *18.2*	0	0	9 *25*	3 *8.8*	7 *9.6*	4 *20*	3 *7.5*	7 *21.2*	19 *18.8*	15 *12.3*	15 *10*
EMB agar																					
Pantoea agglomerans[a]	0	0	0	0	0	0	0	3 *13.6*	0	1 *9.1*	0	0	0	0	0	0	0	0	1 *1*	3 *2.5*	0
Serratia marcescens[a,b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6 *30*	4 *10*	0	6 *5.9*	4 *3.3*	0
Serratia plymuthica[a]	0	0	0	0	0	0	0	0	0	1 *9.1*	0	0	0	0	0	0	0	0	1 *1*	0	0
Tryptic soya agar																					
Aeromonas hydrophila	0	0	1 *7.7*	0	0	0	0	0	0	0	0	0	0	0	0	0	1 *2.5*	0	0	1 *0.8*	1 *0.7*
Burkholderia cepacia	0	0	1 *7.7*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 *0.7*
Chromobacterium violaceum[b]	0	0	0	0	0	0	0	0	0	0	0	0	1 *2.8*	4 *11.8*	0	0	0	0	1 *1*	4 *3.3*	0
Pseudomonas aeruginosa[b]	0	1 *20*	0	0	0	0	4 *25*	0	0	0	0	0	0	0	0	0	0	0	4 *4*	1 *0.8*	0
Stenotrophomonas maltophilia[b]	0	0	0	0	1 *10*	0	0	0	1 *10*	0	0	0	0	0	0	0	0	0	0	1 *0.8*	1 *0.7*

♀♀ = females, ♂♂ = males, NN = nymphs. In the upper part of each cell the minimal number of infected ticks is given, and in the lower part (italic) the calculated minimum infection rate (MIR, %). [a]Primary growth also on tryptic soya agar. [b]Primary growth also on BCYE-α agar.

isolated on EMB agar also showed an ability to grow on TSA, whereas some of the species isolated on TSA showed an ability to grow on BCYE-α agar (Tab. 1).

Strains identified as belonging to *Pasteurella pneumotropica*/*haemolytica* complex proved to be the most common Gram-negative bacteria occurring in *Ixodes ricinus* ticks. They were found in ticks from all the examined woodlands. *Pasteurella pneumotropica*/*haemolytica* was isolated more often from female and male ticks, infected in 5 out of 6 woodlands each, than from nymphs, infected in 3 out 6 woodlands. On average, the minimum infection rate (MIR) in females was 18.8%, in males 12.3%, and in nymphs 10%.

Other species of Gram-negative bacteria were isolated less frequently. Four of them (*Aeromonas hydrophila*, *Pantoea agglomerans*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*), were found in ticks from 2 woodlands and 4 (*Burkholderia cepacia*, *Chromobacterium violaceum*, *Serratia marcescens*, *Serratia plymuthica*) in ticks from 1 woodland. Six isolations were made from males, 5 from females, and 3 from nymphs. Minimum infection rates were low, ranging from 0.7-5.9% (Tab. 1).

DISCUSSION

In the course of bacteriological examination of *Ixodes ricinus* ticks from eastern Poland, the common occurrence of *Pasteurella* strains was found, not reported earlier from any of the hard ticks (Ixodidae) [4, 8, 15, 16, 17, 18, 20]. Until recently, only Martin & Schmidtmann [9] isolated a single strain determined as *Pasteurella* species B from *Ixodes scapularis* female, collected from deer in the USA. The strains determined in this study as *Pasteurella pneumotropica*/*haemolytica* were isolated from ticks collected in all examined localities, and their total number (49) exceeded the total number of all other strains of Gram-negative bacteria belonging to 8 species (30) recovered from these ticks.

The taxon *Pasteurella pneumotropica*/*haemolytica* used in the API identification system comprises 2 related species: *Pasteurella pneumotropica* and *Pasteurella* (*Mannheimia*) *haemolytica*. The inability of the determination of single *Pasteurella* species is a limitation of the API system, and in consequence, also of this study. Nevertheless, both *Pasteurella pneumotropica* and *Pasteurella haemolytica* are known as causative agents of disease in animals and occasionally in humans [1, 6, 7, 11, 24, 25]. Accordingly, even at the present identification level, the results of this study indicate a potential risk of pasteurellosis transmission by ticks.

Pasteurella (*Mannheimia*) *haemolytica* is an important cause of pneumonia in sheep and cattle [1, 6], while *Pasteurella pneumotropica* causes respiratory infection mostly in rodents [7]. Both species may cause infections

in humans [7, 11, 13, 14, 24], which are rare and usually do not involve the respiratory system [25]. Human infection with *Pasteurella pneumotropica* is often transmitted by bites of animals, mostly dogs and cats [7, 11, 14], similar to the well-known case of *Pasteurella multocida* [16]. The ease of *Pasteurella* transmission through injured skin suggests that such transmission could be possible also by tick bite. To check the potential significance of our findings for epidemiology of pasteurellosis in humans and animals, a continuation of this study is planned, including determination of *Pasteurella* isolates with the use of molecular methods.

Of the 9 species of Gram-negative bacteria isolated from *Ixodes ricinus* in the course of the present work, 4 species had been isolated from ixodid ticks by earlier authors: *Burkholderia cepacia* by Murrell *et al.* [12] from *Ixodes holocyclus* in Australia, *Pantoea agglomerans* (synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*) by Murrell *et al.* [12] from *Boophilus microplus* in Australia, and by Dutkiewicz [5] from *Dermacentor reticulatus* in Poland, *Pseudomonas aeruginosa* by Amoo *et al.* [2] from *Boophilus decloratus* and *Boophilus geigyi* in Nigeria, and *Stenotrophomonas maltophilia* by Martin and Schmidtmann [9] from *Ixodes scapularis* in the USA and by Murrell *et al.* [12] from *Ixodes holocyclus*, *Aponomma fimbriatum* and *Boophilus microplus* in Australia. To the best of our knowledge, the isolation of *Aeromonas hydrophila*, *Chromobacterium violaceum*, *Pasteurella pneumotropica*/*haemolytica*, *Serratia marcescens* and *Serratia plymuthica* from ixodid ticks has not been reported previously.

Besides *Pasteurella pneumotropica*/*haemolytica*, at least 3 other species of Gram-negative bacteria isolated from *Ixodes ricinus* in the course of the present work may cause infectious disease in humans: *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, and *Serratia marcescens*. *Chromobacterium violaceum* causes rare, but potentially fatal human infections in tropical and subtropical areas [10]. *Pseudomonas aeruginosa* and *Serratia marcescens* are known causative agents of purulent infections in man [25]. It is noteworthy that while *Pasteurella pneumotropica*/*haemolytica* was commonly distributed over the whole study area, *Chromobacterium violaceum* and *Serratia marcescens* showed a focal distribution and were isolated only from one locality each.

CONCLUSION

The strains of *Pasteurella*, identified as *Pasteurella pneumotropica*/*haemolytica*, proved to be the commonest Gram-negative bacteria occurring in *Ixodes ricinus* ticks collected in eastern Poland. This poses a potential risk of pasteurellosis for humans and animals exposed to tick bites in this area.

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