Research Article

The molecular detection of *Anaplasma phagocytophilum* and *Rickettsia* spp. in cat and dog fleas collected from companion animals

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Abstract: Companion animals can be infested by various species of parasitic insects. Cat flea *Ctenocephalides felis* (*C. felis felis*) (Bouché, 1835) and dog flea *Ctenocephalides canis* (Curtis, 1826) belong to multithost external parasites of mammals, which most frequently occur on domestic cats *Felis catus* Linnaeus and dogs *Canis familiaris* Linnaeus. The main aim of this study was to investigate the presence of pathogens, such as *Anaplasma phagocytophilum* (syn. *Ehrlichia phagocytophila*) and *Rickettsia* spp., in adult *C. felis* and *C. canis* fleas. Flea sampling has been realised from January 2013 to April 2017 in veterinary clinics, animal shelters and pet grooming salons. Fleas were collected from domestic cats and dogs, directly from the pet skin or hair. Then, the DNA was isolated from a single flea by using the alkaline hydrolysis and samples were screened for the presence of pathogens using PCR method. *Anaplasma phagocytophilum* has occurred in 29% of examined *C. felis* and 16% of *C. canis* individuals. In turn, the prevalence of *Rickettsia* spp. in cat fleas population was only 3%, and the dog fleas 7%. The present study showed the presence of pathogenic agents in cat and dog fleas, which indicates the potential role of these insects in circulation of *A. phagocytophilum* and *Rickettsia* spp. in the natural habitat. Furthermore, exposition to these flea species, whose hosts are domestic cats and dogs, can pose a potential risk of infection for humans.

Keywords: *Ctenocephalides felis*, cat flea, *Ctenocephalides canis*, dog flea, *Rickettsia*, PCR

The increased mobility of both human and companion animal populations caused the spreading of many zoonotic pathogens to other areas (Bitam et al. 2010). Hematophagous arthropods, like fleas, play a significant role as vectors in this process. Fleas are obligatory external parasites and multithost insects of medical and veterinary importance (Gray et al. 2009, Torina et al. 2013). They are able to transmit pathogenic species in a variety of ways, such as by blood sucking, horizontal (co-feeding), vertical and mechanical transmission or by infected excrements (Dobler and Pfeffer 2011, Brown et al. 2015, Brown and Macaluso 2016). They can be reservoirs for many bacteria, such as *Yersinia pestis*, * Bartonella henselae*, *B. quintana*, *B. koehleriae*, *B. claridgeiae*, *B. vinsonii* subsp. *berkhoffii*, *B. elizabethae*, *B. rochalimae*, *Rickettsia* spp. or *Francisella tularensis* (see Bechah et al. 2008, Bitam et al. 2010, Víchová et al. 2018).

The presence and possibility of transmission of *Anaplasma phagocytophilum* have been poorly investigated in fleas. So far, the occurrence of this rickettsia has been reported on four continents – Europe, North America, Africa and Asia, mostly in *Ixodes* ticks (Derdakova et al. 2003, Stańczak et al. 2004, Stuen 2007, Radziejwska et al. 2018, Woldehiwet 2010). This species is classified as an opportunistic pathogen, responsible for the induction of granulocytic anaplasmosis, a zoonotic disease causing unspecific symptoms in humans and animals (Dumler et al. 2005).

The occurrence of some species of *Rickettsia* was described in fleas in several European countries (Brouqui et al. 2006, Parola 2011, Hornok et al. 2014, Spitalská et al. 2015, Radziejwska et al. 2018, Viechová et al. 2018). Four groups belong to *Rickettsia*: Spotted Fever Group rickettsiae (SFG) including approximately 20 species, Typhus Group (TG) with *R. prowazekii* and *R. typhi*, the ancestral group (*R. canadensis* and *R. bellii*) and the transitional group (*R. akari*, *R. felis* and *R. australis*) (Wood and Artsob 2011, Radziejwska et al. 2018).

The most frequently occurring rickettsiae in Central Europe are species associated with mites, such as *R. slovaca*, *R. helvetica* and *R. akari* (Raoult and Roux 1997, Viechová et al. 2018). However, a flea-borne species, *R. felis*, which can be transmitted transovarially and transstadially within the most competent vector, cat flea, is also reported from European countries (Gilles et al. 2008, Pérez-Osorio et al. 2008, Capelli et al. 2009, Reif and Macaluso 2009, Lappin 2018).

Most rickettsial infections manifested by non-specific symptoms, such as fever, headache, myalgia, lymphadenopathy, rash; during the chronic form, patients may ex-
Table 1. The number and percentage of *Ctenocephalides felis* (Bouché, 1835) infected with *Anaplasma phagocytophilum* and *Rickettsia* spp. collected from pets in southern Poland.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of studied specimens</th>
<th>No. of <em>Anaplasma phagocytophilum</em> positive fleas (prevalence)</th>
<th>No. of <em>Rickettsia</em> spp. positive fleas (prevalence)</th>
<th>Total no. of infected cat fleas (prevalence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>93</td>
<td>28 (30%)</td>
<td>3 (3%)</td>
<td>31 (33%)</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>3 (23%)</td>
<td>0</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>31 (29%)</td>
<td>3 (3%)</td>
<td>34 (32%)</td>
</tr>
</tbody>
</table>

Table 2. The number and percentage of *Ctenocephalides canis* (Curtis, 1826) infected with *Anaplasma phagocytophilum* and *Rickettsia* spp. collected from pets in southern Poland.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of studied specimens</th>
<th>No. of <em>Anaplasma phagocytophilum</em> positive fleas (prevalence)</th>
<th>No. of <em>Rickettsia</em> spp. positive fleas (prevalence)</th>
<th>Total no. of infected dog fleas (prevalence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>33</td>
<td>7 (21%)</td>
<td>2 (6%)</td>
<td>9 (27%)</td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>0</td>
<td>1 (9%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>7 (16%)</td>
<td>3 (7%)</td>
<td>10 (23%)</td>
</tr>
</tbody>
</table>

Sampling fleas

The collection of fleas was realised from January 2013 to April 2017 in veterinary clinics, animal shelters and pet grooming salons located in the cities of Upper Silesia region in southern Poland, Central Europe. Fleas were collected from domestic cats and dogs, directly from the pets skin or hair. The material was conserved in plastic tubes with 70% ethyl alcohol. Then, collected fleas were determined to species and sex using stereoscopic microscope SZ-40 (Olympus, Japan), according to morphological key of Skuratowicz (1967).

DNA extraction and molecular detection of pathogens

DNA was isolated from a single flea by using the ammonia method (alkaline hydrolysis) (Rijpkema et al. 1996). Individuals were placed in separate sterile plastic tubes with 100 μl of 0.7M NH₄OH. Subsequently, fleas were mechanically crushed by the homogenate CAT X 120 (Ingenieurbüro CAT, M. Zipperer GmbH, Staufen, Germany) and the samples were boiled in a heating block TB-941U (JWElectronic, Warsaw, Poland) at 100°C for 15 min. Then, lids were opened and the samples were boiled at 100°C for 10 min in order to remove the ammonia. They were centrifuged for 5 min at 12,000 rpm and the supernatant was transferred to a new plastic tube. The DNA concentration was measured using the Nanospectrophotometer Pearle (Implen, Munich, Germany). All measurements of the DNA concentration were in ng/μl. The DNA samples were screened for the presence of pathogens using PCR method. The amplification reactions were conducted in a thermal cycler MJ Mini (BioRad, Hercules, CA, USA). A pair of primers EHR521/EHR747 specific to the gene fragment 16S rRNA (Grzeszczuk et al. 2005, Wójcik-Fatla et al. 2009) was used for detection of *Anaplasma phagocytophilum*. The control sample was used thanks to A. Wójcik-Fatla from the Witold Chodzko Institute of Rural Health in Lublin. The conditions of the PCR reaction were as follows: preliminary denaturation at 94°C for 5 min, then denaturation at 94°C for 45 s, annealing at 60°C for 45 s, elongation at 72°C for 20 s and final elongation at 72°C for 7 min. Forty cycles of PCR reaction were performed. For detection of *Rickettsia* spp., a pair of primers RpCs.877p/RpCs.1258n, specific to the gene gltA encoding citrate synthase was used (Regnery et al. 1991, Stańczak et al. 2008). Positive control of *Rickettsia helvetica* was kindly provided by J. Stanczak from the Department of Tropical Medicine and Parasitology from the Medical University in Gdansk. The conditions of the PCR amplification were as follows: initial denaturation at 95°C for 3 min, then denaturation at 94°C for 20 s, annealing at 48°C for 30 s, elongation at 60°C for 2 min and final elongation at 72°C for 7 min. Thirty-five cycles were performed. The PCR products were separated electrophoretically in 2% ethidium bromide-stained gels at 80V for about 2 h. Then the gels were visualised under ultraviolet light and photographed in the analyser Omega 10 (Ultra-Lum, Temecula, CA, USA). The presence of reaction products with the size of 274 base pairs (bp) for *A. phagocytophilum* and 381 bp for *Rickettsia* spp. were considered positive.

Statistical analysis

To compare the frequency of infection by *A. phagocytophilum* and *Rickettsia* spp. in examined flea samples the Yate’s corrected chi-square ($\chi^2$) test was used (Statistica 10.0, PL version).

RESULTS

Identification fleas

In total 155 fleas were collected from the same number of companion animals, such as domestic dogs (*Canis familiaris* Linnaeus) (n = 89) and domestic cats (*Felis catus* Linnaeus) (n = 66). The morphological analysis showed species variability of collected material because four flea species were identified from collected material. The most frequent species was *Ctenocephalides felis* (~68%), followed by *Ctenocephalides canis* (~28%), *Pulex irritans* Linnaeus, 1758 (1.9%) and *Archaeopsylla erinei* (Bouché, 1835) (1.3%). Amongst all collected fleas the majority constituted females (85%). Males were also found, both in *C. felis* and *C. canis* populations.
Detection of pathogenic agents in fleas

In this study all collected cat fleas (C. felis) and dog fleas (C. canis) were molecularly analysed to detect A. phagocytophilum and Rickettsia spp. The DNA was isolated from 150 fleas, including 106 individuals of C. felis (93 females and 13 males) and 44 of C. canis (33 females and 11 males). Anaplasma phagocytophilum occurred in 29% of C. felis individuals. The presence of this rickettsia was confirmed both in females and males of analysed cat fleas (Table 1). The prevalence of this species in C. canis was 16% and A. phagocytophilum was detected only in females (Table 2). The prevalence of Rickettsia spp. in examined C. felis population was 3% (Tab. 1). This species was more often detected in dog fleas and it accounted for 7% (Table 2). The presence of Rickettsia spp. was found both in females and males of C. canis and C. felis.

Statistical analysis

The statistical analysis showed that cat fleas were significantly more often infected by A. phagocytophilum than Rickettsia spp. ($\chi^2 = 23; p \leq 0.00001$) (Table 1). In contrast, no significant difference was observed between the number of dog fleas infected by examined species of rickettsiae ($\chi^2 = 3.14; p = 0.0762$) (Table 2). In comparison of the two adult forms of C. canis, females were significantly more often infected by A. phagocytophilum than males ($\chi^2 = 21; p \leq 0.00001$). There was no significant difference between the infection by Rickettsia spp. amongst females and males of C. canis ($\chi^2 = 0.29; p = 0.59$) (Table 2). In case of C. felis, no difference was found for both adult forms in frequency of the A. phagocytophilum ($\chi^2 = 0.92; p > 0.34$) and Rickettsia spp. infection ($\chi^2 = 1.35; p > 0.245$) (Table 1).

DISCUSSION

In the last decades, the attention of scientists has been focused on transmission of many pathogens by arthropods, especially ticks and mosquitoes. The data about fleas and their ability to transmit infectious agents is limited. The main purpose of this study was to investigate the presence of pathogens, such as Anaplasma phagocytophilum and Rickettsia spp., in adult Ctenocephalides felis and Ctenocephalides canis fleas.

The first attempt to detect A. phagocytophilum in fleas was made in the USA, in species of C. felis collected from domestic cats, but with negative results (Lappin et al. 2006). Another study, from Portugal also did not confirm presence of A. phagocytophilum in this flea species (Alves et al. 2009). Torina et al. (2013) showed a low prevalence (1%) of this rickettsia, but only in the Oriental rat flea, Xenopsylla cheopis (Rothschild, 1903). In the same study, other flea species such as Ctenocephalides canis, C. felis and Cediopsylla inaequalis (Baker), were collected from foxes Vulpes vulpes (Linnaeus) and molecularly tested, but with negative results for A. phagocytophilum (see Torina et al. 2013).

In our study, the occurrence of A. phagocytophilum was confirmed both in analysed cat and dog fleas. The prevalence of this species in case of C. felis was at a high level of 29%, whereas in C. canis population 16%. High frequency of A. phagocytophilum in examined fleas can indicate the occurrence of many reservoirs of this species in southern Poland. Moreover, frequent detection of this rickettsia in obligatory parasites of pets, like dog and cat fleas may indicate a high risk of exposure to this pathogen both among animals and their owners in examined area and other places, where human and animal populations migrate.

Another zoonotic pathogen detected in cat and dog fleas collected from pets was Rickettsia spp. In our research, the prevalence of this pathogen was 3% in cat and 7% in dog fleas. According to our knowledge, fleas may take part in natural transmission of R. typhi and R. felis (see Ereneeva et al. 2008, Teoh et al. 2017), as well as R. rickettsii in experimental conditions (Dobler and Pfeffer 2011, Blanton and Walker 2017). A study from the Netherlands showed occurrence of Rickettsia helvetica DNA in two unknown species of fleas collected from rodents (Sprong et al. 2009). Furthermore, Hornok et al. (2014) showed the presence of this species in two females of Archeaopsylla erinacei.

Moreover, Rickettsia spp. were found in 10.8% of analysed fleas Ctenophthalmus agyres (Heller, 1896), C. solutus Jordan et Rothschild, 1920, C. uncinatus (Wagner, 1898) and Nosopsyllus fasciatus (Bose, 1800) in Slovakia (Spitalská et al. 2015) and in 44% fleas collected from rodents C. agyres, Ctenophthalmus assimilis (Taschenberg, 1880), Hystriopsylla talpae (Curtis, 1826), Megabothris turbidus (Rothschild, 1909), M. walkeri (Rothschild, 1902), Palaeopsylla soricens (Dale, 1878) in Lithuania (Radzijevska et al. 2018). There are many studies in Europe which confirmed the presence of Rickettsia felis in fleas (Gilles et al. 2008, Pérez-Osorio et al. 2008, Capelli et al. 2009, Reif and Macaluso 2009, Gracia et al. 2015). According to our knowledge, this is the first study in Poland, which showed the presence of Rickettsia spp. in Ctenocephalides fleas.

In reference to research, which confirms the presence and possibility of transmission other species of rickettsiae, like R. felis and R. typhi in fleas (Pérez-Osorio et al. 2008, Abdad et al. 2011), the vector and reservoir role of examined cat and dog fleas for A. phagocytophilum and Rickettsia spp. could be possible, however future study are required. In spite of conducted studies about transmission of pathogenic agents by fleas, our knowledge is still limited. To explain the role of C. felis and C. canis in transmission of A. phagocytophilum and Rickettsia spp., an experimental study with screening the blood of their hosts or laboratory animals should carried out.

In conclusion, the conducted study revealed the presence of Anaplasma phagocytophilum and Rickettsia spp., both in cat and dog fleas, which indicates the potential role of examined insects in circulation of these pathogens in the natural habitat. Moreover, both C. felis and C. canis belong to fleas with a low host specificity and they are able to bite humans, what make a potential peril of rickettsial infection.

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REFERENCES


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