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First investigations on serum resistance and sensitivity of *Borrelia turcica*

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ABSTRACT

Borrelia turcica is a reptile-associated *Borrelia* species that is vectored by the hard tick *Hyalomma aegyptium*. Tortoises of the genus *Testudo* represent the principal host of adult *H. aegyptium*, while immature stages are less host-specific and can be found on various vertebrates and even on humans. *Borrelia turcica* isolates were already successfully obtained from exotic tortoises suggesting that they are putative hosts. To the best of our knowledge, no further investigations on additional host association of *B. turcica* were conducted. Since many but not all adult *Hyalomma* ticks collected from tortoises are infected, questions arise about the direction of transmission between tick and tortoises for this *Borrelia* species. In addition, there is no information on the potential pathogenicity of *B. turcica* for humans. For other *Borrelia* species it has been shown that resistance or sensitivity to complement-active serum can be indicative of host species association(s). In this study, we explored for the first time the *in vitro* survival of *B. turcica* isolates from Turkey (IST7) and Greece (171601G) in the presence of 50% complement-active serum of different species (tortoise, turtle, human and bird). Both isolates showed resistance to tortoise serum, partial resistance to turtle serum but did not survive human and bird serum. These data suggest that indeed tortoises are reservoir host species for *B. turcica* while birds or humans are not. By implication these data suggest that *B. turcica* is not human pathogenic. Whether or not other reptile species, such as lizards, are also potential hosts, requires further investigation. However, as the life cycle of *Borrelia* is closely linked to that of their hosts and vectors, *in vitro* studies can only give clues about the actual *in vivo* behavior.

1. Introduction

The genus *Borrelia* (*B.*) is divided into the Lyme borreliosis (LB) group, the relapsing fever (RF) group and reptile- or echidna-associated species (Gofton et al., 2018; Loh et al., 2016; Takano et al., 2011). *Borrelia turcica* is reptile host associated that clusters as a sister clade to the RF group and is vectored by the hard tick *Hyalomma* (*H.*) *aegyptium* that has a typical three-host life-cycle (Güner et al., 2004; Siroky et al., 2011). Main host of adult ticks are tortoises of the genus *Testudo* (*T.*). Nevertheless, different *Testudo* species show varying attractiveness to *H. aegyptium*: *Testudo graeca* and *T. marginata* represent the principal host while other species such as *T. hermanni* are reported to be infested very rarely (Siroky et al., 2006) or not at all by these ticks (in this study). Immature stages of *H. aegyptium* are less host-specific and can be found in the field on various vertebrates such as tortoises, lizards, birds, small mammals and even humans (Pastiu et al., 2012) or can be reared in the laboratory on guinea pigs (Siroky et al., 2011). In contrast to *H. aegyptium*, the host association of *B. turcica* has not yet been investigated as this species does not appear to be relevant for public health. Tortoises are thought to be putative hosts, as *B. turcica* was successfully isolated from blood und multiple organs of exotic tortoises (Takano et al., 2010). However, no studies have been conducted on the association of *B. turcica* to species that are hosts for immature stages of *H. aegyptium*. Therefore, it is still unclear whether tortoises infect the ticks feeding on them or whether the tortoises receive their infection through already *Borrelia*-positive adult ticks that were infected during a previous blood meal as larvae or nymph. Furthermore, transovarial transmission - a way of vertically transmitting *Borrelia* (mainly relapsing fever species) in ticks (Barbour, 2014) - has not been confirmed for *B. turcica* (Kalmar et al., 2015).

Host association has an impact on geographical distribution and population structure of *Borrelia* as the migration of ticks and *Borrelia* is

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directly linked to movements of their hosts (Kurtenbach et al., 2002b; Vollmer et al., 2011, 2013). Host association is strongly linked to resistance/ sensitivity to the alternative pathway of the host complement system in *Borrelia* (Kraiczy, 2016; Tufts et al., 2019).The complement system is the major arm of the innate immune system that defends the host against pathogens and leads to damage or lysis of microorganisms (Kurtenbach et al., 2002a; Marcinkiewicz et al., 2017). *Borrelia* can prevent complement-mediated bacteriolysis by binding host regulator proteins. This binding is facilitated by bacterial surface proteins called CRASPs (complement regulator-acquiring surface proteins) (Kraiczy et al., 2001a, b). On the cell surface different combinations of these proteins may be found during the host - vector transmission cycle (Kraiczy and Stevenson, 2013) which probably have an impact on complement resistance and complement sensitivity to serum of different species (Tufts et al., 2019). This in turn plays a key role for host specificity (Kraiczy, 2016).

Previous studies on the population structure of *B. turcica* from Greece and Turkey had shown that *B. turcica* isolates from both regions are phylogenetically closely related (own unpublished data: LGL Oberschleissheim; Hepner, Margos, Fingerle, Duscher et al.). This finding and the host choice of immature *Hyalomma* stages prompted us to investigate how *B. turcica* would react to complement-active serum of potential hosts. The aim was to obtain first insights into potential host associations of *B. turcica*. The *in vitro* behavior of *B. turcica* in presence of complement-active serum of different vertebrate species (tortoise, turtle, human and bird) was analyzed. Our data suggest that tortoises are reservoir hosts while blackbirds and humans are not.

2. Materials and methods

2.1. Borrelia strains

Two *B. turcica* strains (from Turkey and Greece) were included into this study. The Turkish *B. turcica* type strain IST7 was kindly provided by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig). The strain was originally isolated from *H. aegyptium* in 2003 in the Istanbul area in northwestern Turkey (Güner et al., 2003, 2004). The Greek isolate (171601G) was obtained from a feeding *H. aegyptium* collected from a wild *T. graeca* in Greece near Serres in 2017 and cultivated in modified BSK medium (see below). The strain was identified as *B. turcica* by multilocus sequence typing (MLST) (Margos et al., 2008, 2009). Used primer sequences (adapted for *B. turcica*) and running conditions are listed in supplementary information (Table A.1).

Two Lyme borreliosis species were used as positive controls to determine serum complement activity. *Borrelia bavariensis* (PBi), which is associated with rodents (Huegli et al., 2002; Margos et al., 2009), was used to test the complement activity *in vitro* of tortoise, turtle and bird serum. All these sera, if complement-active, should result in lysis of *B. bavariensis*. As positive control for complement activity of human serum, the human complement-sensitive species *B. lusitaniae* (PotiB2) (Kraiczy, 2016) was employed.

2.2. Preparation of serum

The Clinic of Birds, Small Mammals, Reptiles and Ornamental Fish of the Ludwig-Maximilian University Munich (Faculty of Veterinary Medicine) in Oberschleissheim implements propaedeutic courses for clinical activity in rotation (ethical permission granted; number of the animal experiment application: ROB-55.2Vet-2532.Vet_03-17-86). In this course blood samples of tick-free tortoises (*T. hermanni*) and turtles (*Trachemys scripta elegans*) were taken. The blood was kindly provided for preparation of complement-active serum. The serum was prepared as described by Lachmann (Lachmann, 2010). Briefly, blood was collected in absence of anticoagulants in a glass tube and clotted at room temperature. After a centrifugation step (3,000 x g, 10 min, room temperature) the supernatant was transferred in a new tube and centrifuged a second time (20,000 x g, 4 min, room temperature). The supernatant was aliquoted and stored at - 80 °C. Blackbird (*Turdus merula*) serum was kindly provided from the University of Antwerp (Department of Biology), obtained from non-tick exposed animals. *Borrelia* antibody negative human serum was purchased from Biomex, Germany (www.biomex.de). All sera were stored at - 80 °C immediately after arriving and thawed rapidly at the time for use in a 37 °C water bath. Serum samples were maximum once thawed, aliquoted and refrozen immediately. Tortoise and turtle serum was aliquoted and immediately frozen. For complement inactivation, serum was incubated in a 56 °C water bath for 30 min.

2.3. In vitro cultivation and complement assay of Borrelia strains

Borrelia turcica isolates were cultured in modified BSK medium (BSK-Y). Medium compositions are shown in supplementary information Table A.2. Modified MKP medium was used for cultivating LB group *Borrelia* species. Basal MKP medium (Preac-Mursic et al., 1986) was completed with autoclaved gelatin solution (Merck Art. 4070), rabbit serum (Gibco No. 037-06120) and bovine serum albumin (Sigma No. A-7409) at a final concentration of 15%, 5% and 5%, respectively. Isolates were cultured in 96-well microplates (Edge BioSystems Art. 28777) at a temperature of 33 °C and under 5% CO_2 atmosphere.

Borreliacidal assays were performed similar to the ones implemented and described previously by Kurtenbach et al. (1998). *Borrelia* cultures were grown to E + 06 - E + 07 cells/ mL medium. Experimental samples were done in triplicates and consisted of 25 µL *Borrelia* culture and 25 µL serum (samples and positive control)/ 25 µL inactivated serum (negative control). Because of the limited amount of bird serum, we performed only one test with IST7, two with 171601 G and we used medium instead of inactivated serum as negative control. Cell densities were determined at the start (0 h) of the complement test and 24 h later as described previously (Rößle, 2001). Briefly, 13 µL of the culture was used, placed on a slide and covered with a coverslip (24 x 60 mm). The number of *Borrelia* per field were determined for 30–50 fields using dark field microscopy (eyepiece magnification 10x, objective magnification 40x). For calculations of the cell density in the culture (*Borrelia*/ mL), we used following formula: (total number of counted cells * 3,500 * 1,000) / (number of counted fields * 3). After 2 h the cultures were examined microscopically for vitality (structure, bleb formation, motility) using dark field microscopy (magnification see above).

3. Results

To test the complement resistance/ sensitivity of two *B. turcica* isolates (IST7 and 171601G) *in vitro* cultures were supplemented with complement-active serum of different species (tortoise, turtle, human and bird). Cell densities and survival were determined at 0 h and 24 h (Table 1A (0 h) and Table 1B (24 h)) and cultures were microscopically examined after 2 h. Densities of samples cultivated in the presence of complement-active and heat-inactivated serum (negative controls) are shown in Fig. 1A (IST7) and Fig. 1B (171601G). Error bars are only included at time point 24 h, as only here triplicate counts were performed. To set up experiments, a single culture was counted and all experimental wells received the same amount of culture. All positive controls for complement activity (PBi, PotiB2) were completely lysed by 24 h and no surviving bacteria were found in the cultures, thus, complement activity was confirmed (Table 1, not shown in Fig. 1). In all negative controls (only one culture) the cell density increased and vitality of *Borrelia* was high. Cell densities of negative controls (exception: inactivated tortoise serum) of IST7 were higher than that of 171601G. Only in the presence of complement-active tortoise serum was an increase of the cell densities of both samples (IST7 and 171601G) observed. The density of the Turkish strain (IST7) increased by a factor ten within 24 h (from 3.2E + 06 *B.*/ ml medium to 3.6E + 07 *B.*/ ml

Table 1
Borrelia cell density in *Borrelia/* ml medium in presence of different sera (tortoise, turtle, human and bird) at experiment start (0 h, Table 1A) and after 24 h (Table 1B). pc = positive control, nc = negative control. For nc cultures were cultivated with inactivated serum (exception: nc for bird serum was run with medium instead of inactivated serum).

A

0 h	IST7_1	IST7_2	IST7_3	171601G_1	171601G_2	17601G_3	pc serum	nc IST7	nc 171601G
tortoise	3.2E + 06	3.2E + 06	3.2E + 06	4.3E + 06	4.3E + 06	4.3E + 06	6.4E + 06	3.2E + 06	4.3E + 06
turtle	3.2E + 06	3.2E + 06	3.2E + 06	4.3E + 06	4.3E + 06	4.3E + 06	6.4E + 06	3.2E + 06	4.3E + 06
human	3.2E + 06	3.2E + 06	3.2E + 06	4.3E + 06	4.3E + 06	4.3E + 06	6.4E + 06	3.2E + 06	4.3E + 06
bird	3.2E + 06	–	–	4.3E + 06	4.3E + 06	–	6.4E + 06	3.2E + 06	4.3E + 06

B

24 h	IST7_1	IST7_2	IST7_3	171601G_1	171601G_2	171601G_3	pc serum	nc IST7	nc 171601G
tortoise	4.0E + 07	4.0E + 07	2.9E + 07	1.6E + 07	2.3E + 07	1.6E + 07	0.0E + 00	4.1E + 07	4.6E + 07
turtle	1.9E + 05	2.3E + 05	2.8E + 05	4.3E + 06	2.7E + 06	3.1E + 06	0.0E + 00	2.9E + 07	1.3E + 07
human	0.0E + 00	0.0E + 00	0.0E + 00	0.0E + 00	0.0E + 00	0.0E + 00	0.0E + 00	2.9E + 07	1.3E + 07
bird	0.0E + 00	–	–	0.0E + 00	0.0E + 00	–	0.0E + 00	3.0E + 07	2.1E + 07

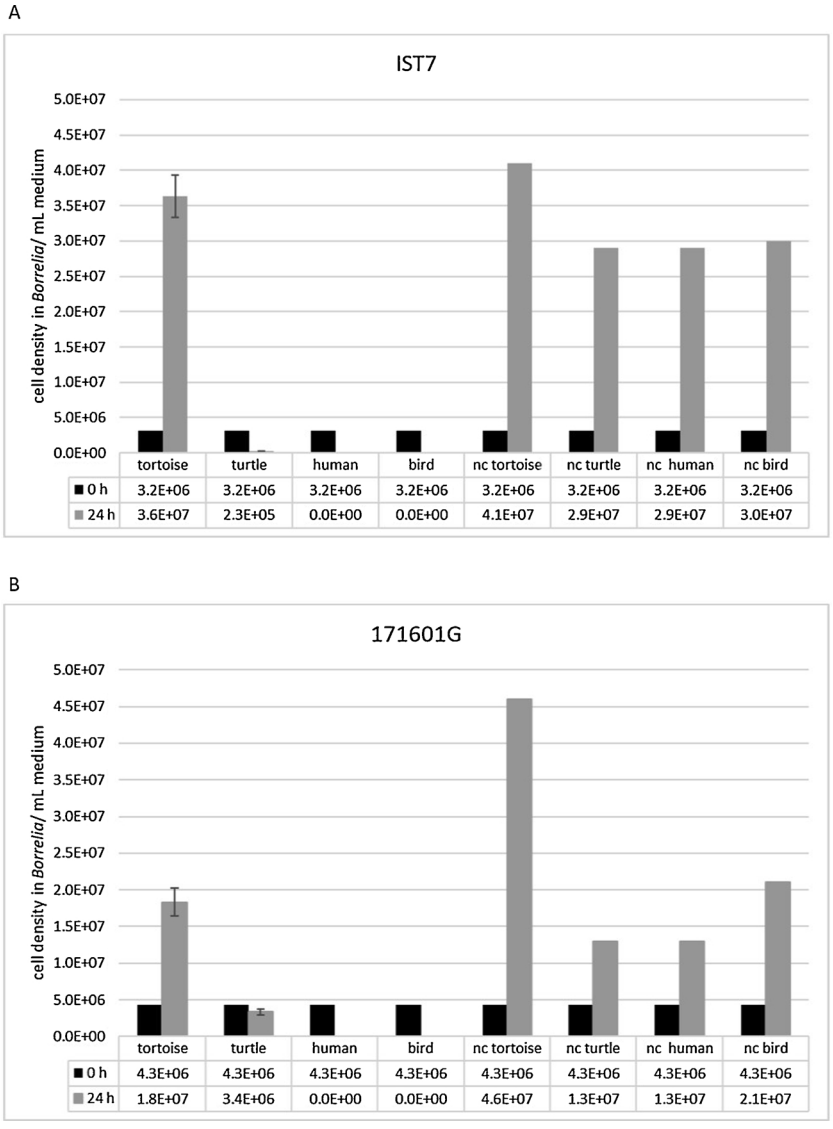

Fig. 1. Cell densities in *in vitro* cultures of *B. turcica* exposed to complement-active serum or heat-inactivated serum or medium (nc) of tortoise, turtle, human and bird. IST7 (A) and 171601G (B) at start of the complement test (0 h, black) and 24 h later (grey). Both isolates survived only in tortoise and turtle serum. In human and bird serum, only dead borreliae were found after 24 h. Good borreliae vitality and growth was observed in the negative controls (nc). See Table 1 for details.

medium) and was comparable to its negative control. The Greek strain (171601G) also showed cell growth in active tortoise serum (from 4.3E +06 *B./* ml medium to 1.8E+07 *B./* ml medium) but less than IST7 and its negative control. In both cultures high motility and good cell structure were found after 2 h and 24 h and no adverse effect of the presence of complement was observed. Therefore, *B. turcica* seems to be resistant to tortoise complement-mediated killing. Regarding the turtle serum an initial decrease in cell density was observed after 2 h. Interestingly, at this time point most of the cells were dead, only few *Borrelia* cells with good motility (171601G) or at least twitching cells (IST7) were observed. However, after 24 h surviving cells had increased in density, with 171601G showing a substantial higher density (3.4E+06 *B./* ml medium) than IST7 (2.3E+05 *B./* ml medium) but densities in both test samples were lower than the negative controls and start density. This is suggestive of an intermediate turtle serum resistance. The human serum and the bird serum completely lysed all cells in the cultures, indicating sensitivity of *B. turcica* to these sera.

4. Discussion

The complement system is part of the innate immunity of vertebrates (including humans, birds and reptiles) (Nonaka and Yoshizaki, 2004; Sunyer et al., 2003) and plays a key role in host association of *Borrelia* (Kurtenbach et al., 2002a). There are three activation routes: classical pathway, the lectin and the alternative pathway (Kraiczy, 2016; Kurtenbach et al., 2002b). Latest can be directly activated through cell surface and revealed as the lytic component in serum (Kurtenbach et al., 1998). The aim of this study was to analyze the *in vitro* behavior of *B. turcica* originating from different geographic regions (Greece and Turkey) and to identify potential hosts for this reptile-associated species. Our results showed that both *B. turcica* strains were lysed by human and blackbird serum, suggesting complement sensitivity for these sera. As previous data have shown a strong match between reservoir host competence and complement sensitivity, the data further suggest that these vertebrates are not reservoir hosts for *B. turcica* and that this reptile-associated species is not human pathogenic. An earlier study reported the successful isolation of *B. turcica* from exotic tortoises (Takano et al., 2010) and our study confirmed that *B. turcica* is resistant to complement-mediated killing by tortoise serum. Taken together these data reinforce the position of tortoises as potential reservoir hosts.

We noticed that IST7 showed better survival (*i.e.* a higher cell density) being exposed to tortoise complement-active serum than 171601G. However, cells in both cultures showed excellent vitality, motility and structure. Interestingly, in both cultures the presence of turtle serum initially decreased the cell density (after 2 h) but subsequently cell numbers increased again (after 24 h). However, they were still lower than at the beginning of the experiment or the negative controls suggesting a negative effect of turtle complement on cell survival. Therefore, *B. turcica* showed intermediate serum resistance/sensitivity to turtle serum. Nevertheless, a difference in *Borrelia* vitality was observed after 2 h: 171601G showed better vitality (good motility of some cells) than IST7 (only twitching). As a result, the cell density of 171601G after 24 h was nearly a factor 15 higher than IST7 but did not reach the density in negative controls. Clearly, the turtle serum had a negative effect on *B. turcica.* We have shown in this study that *B. turcica* is resistant to tortoise serum (*T. hermanni*) but only partially resistant to turtle serum (*Trachemys scripta elegans*). These animals show differences in their systematic classification (family *Testudinidae vs* family *Emydidae*), ecology (land animals *vs* water adapted) and distribution (southern Europe *vs* American continent, respectively) (www.reptile-database.org). We can only speculate here that the differences reflected by these criteria had led to divergence of complement component and therefore cause differences in serum resistance of *B. turcica*.

In this study we have shown that complement lysis of only tortoise and turtle serum was overcome by *B. turcica* from Greece and Turkey. Therefore, only these reptile species appear to be potential reservoir hosts. However, only serum of one bird species (blackbird) was tested and we have not tested either rodent or lizard serum in this study which are also hosts for immature *H. aegyptium*. Given that, it is still unclear whether tortoises get infected by the adult ticks or tortoises infect these ticks. So far, there is no indication that *Borrelia* infection of immature stages of *H. aegyptium* is due to transovarial transmission or whether further putative reservoir hosts for *B. turcica* exist in nature and further studies are required to investigate this. Furthermore, *in vitro* behavior is actually only an indication of *in vivo* behavior as there is a complex interplay between host, vector and *Borrelia* (Kraiczy, 2016).

To understand the biology of the reptile-associated species *B. turcica*, further investigations concerning host and vector association are needed. This might include further studies on serum resistance and sensitivity (including more species *e.g.* further bird species, rodent and lizard), experimental transmission studies including different hosts or vectors and different stages of the tick vectors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.06.013.

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