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SHORT COMMUNICATION

Tortoise tick *Hyalomma aegyptium* as long term carrier of Q fever agent *Coxiella burnetii*—evidence from experimental infection

Pavel Široký · Michaela Kubelová · David Modrý · Jan Erhart · Ivan Literák · Eva Špitalská · Elena Kocianová

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Abstract The experimental study investigated the ability of tortoise tick *Hyalomma aegyptium* to play a role in forming and maintaining natural foci of Q fever. We tested the competence of *H. aegyptium* larvae to acquire *Coxiella burnetii* infection from mammals, serve as a *C. burnetii* vector between mammalian hosts, and be a long-term carrier of *C. burnetii*, including interstadial transmission. *H. aegyptium* larvae were allowed to feed on guinea pigs experimentally infected with *C. burnetii*. Engorged larvae molted to nymphs, some of which were preserved in 96% ethanol and later examined by polymerase chain reaction (PCR) using *C. burnetii*-specific primers (CBCOS, CBCOE). Prevalence of *C. burnetii* among these nymphs was 5.6% (n=235). Remaining nymphs then fed on other, *C. burnetii*-negative guinea pigs; and according to results of both, micro-agglutination reaction, and ELISA, they successfully transmitted *C. burnetii* to those new hosts. Detached engorged nymphs molted to adults, which were kept alive long term and then placed in 96% ethanol 383 days post-infection. Thereafter, they were examined by PCR in the same manner as were the nymphs. Prevalence of *C. burnetii* among adult *H. aegyptium* was 28.9% (n=90). According to our results, tortoise-specific ticks have indisputable potential in the epidemiology of Q fever natural foci.

P. Široký (✉) · M. Kubelová · I. Literák
Department of Biology and Wildlife Diseases,
Faculty of Veterinary Hygiene and Ecology,
University of Veterinary and Pharmaceutical Sciences,
Palackého 1–3,
612 42 Brno, Czech Republic
e-mail: sirokyp@vfu.cz

D. Modrý
Department of Parasitology, Faculty of Veterinary Medicine,
University of Veterinary and Pharmaceutical Sciences,
612 42 Brno, Czech Republic

D. Modrý · J. Erhart
Institute of Parasitology, Biology Center,
Academy of Sciences of the Czech Republic,
Branišovská 31,
370 05 České Budějovice, Czech Republic

E. Špitalská · E. Kocianová
Institute of Virology, Slovak Academy of Sciences,
Dúbravska cesta 9,
842 45 Bratislava, Slovakia

Introduction

Coxiella burnetii, the etiological agent of Q fever, is a widely distributed obligatory intracellular Gram-negative parasite belonging to the gamma subdivision of Proteobacteria (Weisburg et al. 1989). It is known to be responsible for infections, particularly in mammals, including man, and it possesses high zoonotic potential (e.g., Hirai and To 1998; Marrie and Raoult 1997; Reusse 1960). *C. burnetii* has complicated developmental cycle and creates both large and small cell variants (SCV), with the latter of low metabolic and replication activities and are capable of great environmental stability. SCV can persist for a long time in soil and dust, contaminate surface water, and can be spread over distance of several kilometers (Lukáčová et al. 2002).

C. burnetii is dispersed through animal excretions, particularly by females, through decidua, colostrum, and milk (Hirai and To 1998; Marrie and Raoult 1997). Ticks play an important role in the circulation of *C. burnetii* within Q fever natural foci, however, and they are considered primary vectors among wild animals (Burgdorfer and Varma 1967). Ticks transmit *C. burnetii* vertically

(transstadially and transovarially) and horizontally (by biting via saliva; Řeháček and Brezina 1968) but also through ticks' feces, which represent the most concentrated natural source of *C. burnetii* (up to 10^{12} infectious organisms per gram; Hirai and To 1998).

Natural or experimental *C. burnetii* infection has been reported for more than 40 tick species (Parola and Raoult 2001; Řeháček and Tarasevich 1988; Reusse 1960), including *Hyalomma aegyptium* (Blanc 1961), the dominant tortoise tick in the Mediterranean region and in the Middle East. However recently, Socolovschi et al. (2009) report that 140 tick species in 12 genera can carry *C. burnetii*. Although the tortoises of the genus *Testudo* are the principal hosts for adults of *H. aegyptium*, this tick species posses three-host lifecycle, alternating between tortoises, other reptiles and mammals (Apanaskevich 2003; Hoogstraal 1956; Hoogstraal and Kaiser 1960; Široký et al. 2006).

Compared to mammals and birds, little attention has been given to the potential of reptiles and their ticks to play a role in forming natural foci of infectious diseases. Nevertheless, *C. burnetii* has already been reported from reptiles, including turtles and tortoises (Stephen and Achyutha Rao 1979a, b; Yadav and Sethi 1979, 1980). Sharing of the same pastures by tortoises and domesticated ruminants is common in many areas. Nevertheless, no study has yet dealt with the possibility of *C. burnetii*'s transmission between mammals and tortoises by shared developmental stages of their ticks.

At the end of the 1950s, Blanc et al. successfully induced bacteremia in the tortoise *Testudo graeca* by intracardial injection of material rich in *C. burnetii*. *H. aegyptium* ticks allowed to feed on this infected tortoise became long-term carriers of *C. burnetii* (Blanc 1961; Blanc and Ascione 1960; Blanc et al. 1959). Our experimental study was intended to test the capability of *H. aegyptium* (1) to acquire *C. burnetii* infection from infected small mammals, (2) to serve as a vector of *C. burnetii* between mammalian hosts, and (3) to be a long-term carrier of *C. burnetii*, including its interstadial transmission.

Materials and methods

Animals used in experiments, *C. burnetii* strain

Unsexed guinea pigs weighing 450 g and originating from the certified laboratory breeding facility at the Institute of Experimental Pharmacology of the Slovak Academy of Sciences, Dobrá Voda (Certificate No. SK Ch 40004), were used in the experiments. The *H. aegyptium* ticks used originated from the laboratory breeding colony of the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, at České Budějovice. The tick colony had been established from an engorged female imported from Qualat Samaan, Syria, in April 2005. We used an F3 generation hatched under laboratory conditions. *C. burnetii*, strain Nine Mile ($CbNMEP_1$), originated from the Institute of Virology, Slovak Academy of Sciences, Bratislava. It is stored as a 40% suspension of chick embryo yolk sac in saline solution (pH 7.2) at −70°C.

Design of experiments

The experimental study was conducted in a certified facility (BSL 3) of the Institute of Virology, Slovak Academy of Sciences, Bratislava, and was divided into two feeding experiments (Exp1, Exp2; see Fig. 1). Six guinea pigs were used in Exp1. Five of them (labeled as P1–P5) were intraperitoneally infected by 1 ml per animal of the 40% suspension of *C. burnetii* $CbNMEP_1$ in 1:100 dilution. The sixth guinea pig served as a negative control (K1). This specimen was inoculated by placebo (saline solution, pH 7.2). In the same day (day post-infection, dpi 0), 600 *H. aegyptium* larvae were allowed to feed on the experimental guinea pigs. One plastic feeding chamber was glued to the clipped back of each animal, and then ticks were introduced into this chamber, which was immediately covered by dense nylon cloth. Four to 10 days later, the engorged tick larvae detached spontaneously from individual guinea pigs and thereafter were kept separately in 15-ml glass

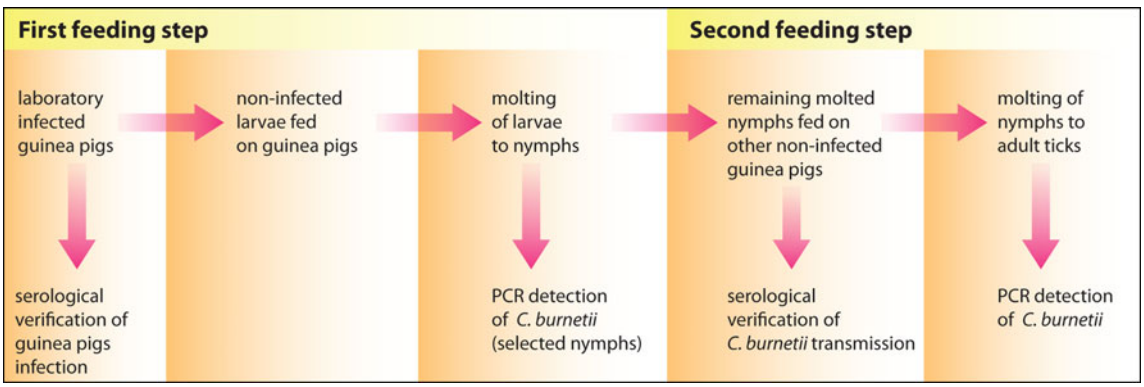

Fig. 1 Schematic design of experimental study

vials with filter paper and closed by cloth bound. Vials with ticks were kept in a plastic box at 23–26°C and relative humidity 60–86%. After the larvae had molted into nymphs, an appropriate number of them from each group (altogether 294 nymphs, Fig. 2) was preserved in 96% ethanol for later DNA isolation and subsequent polymerase chain reaction (PCR) diagnostics.

Twelve weeks after Exp1, the remaining 220 molted nymphs were involved in a second feeding experiment (Exp2), whereby they were allowed to feed on other, *C. burnetii*-negative guinea pigs (P6–P12, K2; see Fig. 1). Engorged detached nymphs were kept in the same manner as were the larvae in Exp1 until they molted into adult ticks. The adults were then kept alive long term and at dpi 383 were individually preserved in 96% ethanol and stored for DNA isolation.

To avoid air borne transmission of infection, all guinea pigs were kept individually in closed boxes, which were placed separately in an Ekostar flow HF-BH laminar box equipped with a ULPA H15 filter (Ekokrok s.r.o., Žilina, Slovakia). They were examined serologically using the micro-agglutination reaction (MAR) and enzyme-linked immunosorbent assay (ELISA) methods for the presence of phase II *C. burnetii* antibodies both before as well as after ticks' engorgement and using the antigen CbNM FII (Institute of Virology, Slovak Academy of Science, Bratislava; Fiset et al. 1969; Kováčová and Kazár 2000; Kováčová et al. 1987). Blood samples for serological tests were collected by intracardial punction under deep anesthesia. In MAR, the titre of sera 1:16 and higher were considered as positive. In ELISA test, titre was considered positive each value 1:500 and higher.

PCR detection of *C. burnetii* DNA

DNA from ticks was extracted using alkaline hydrolysis (Rijpkema et al. 1996). Briefly, the ethanol-preserved ticks were air dried on filter paper, placed separately into vials, crushed with single-use pipette tips, and then boiled for 30 min in 500 µl of 0.36 M ammonium hydroxide in sealed vials to free the DNA. Subsequently, the vials with the lysate were opened and incubated for an additional 20 min at 100°C to remove the ammonium. The tick lysates were stored at −20°C until further use. Negative controls were incorporated into each DNA isolation step to detect possible cross-contamination. Concentration and integrity

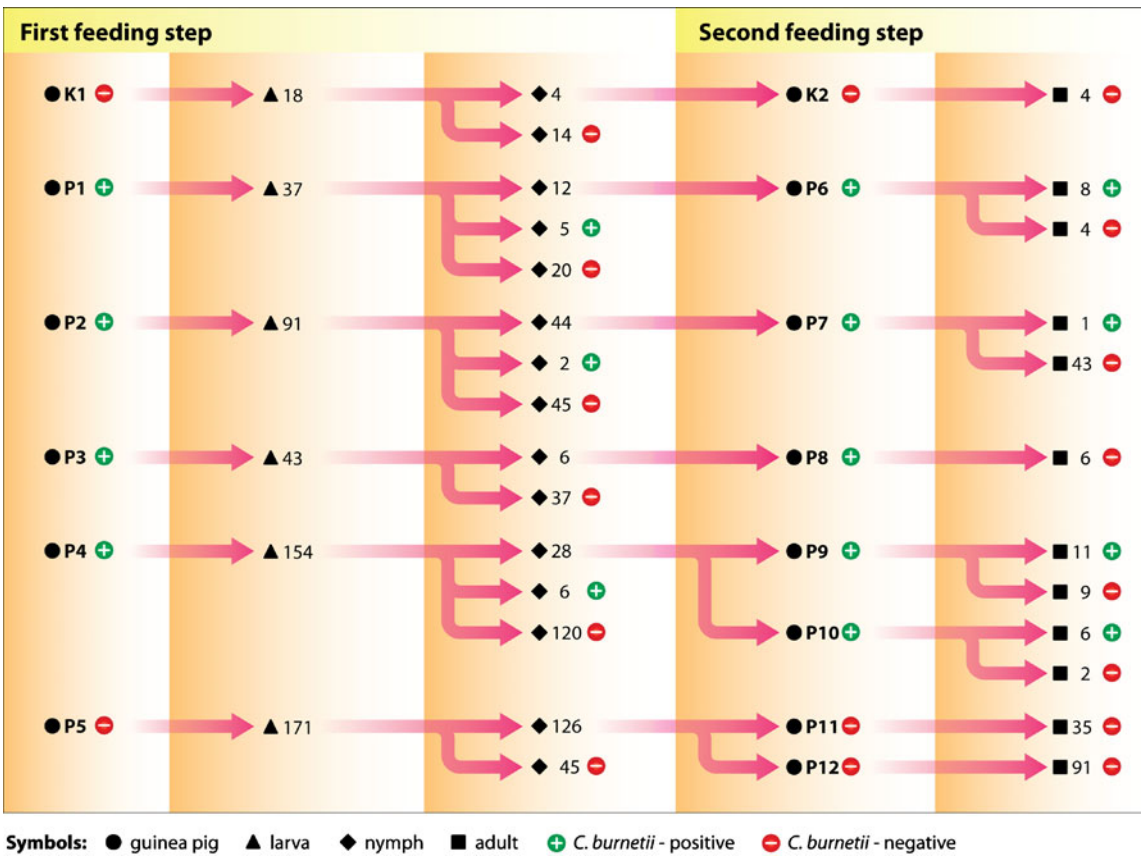

Fig. 2 Schema of results of experimental study. K1, K2, and P1–P12 refer to labeling of guinea pigs used. *Numbers at symbols* present numbers of tick specimens

of the DNA were controlled on an Eppendorf BioPhotometer™ (Eppendorf A.G., Hamburg, Germany) and on agarose electrophoresis gel, respectively.

For *C. burnetii* diagnostics, PCR amplification was performed using oligonucleotide primers CBCOS and CBCOE, which amplify portions of the *com1* gene, encoding an approximately 27-kDa outer membrane associated immunoreactive protein of *C. burnetii* (Hendrix et al. 1993). The size of the expected PCR product is 494 bp (Špitalská and Kocianová 2003). PCR was performed in a 25 μl volume consisting of 12.5 μl of Combi PPP MasterMix (TopBio s.r.o., Prague, Czech Republic), 0.25 pmol of each primer, 9.5 μl of PCR water (TopBio s.r.o., Prague, Czech Republic), and 2.5 μl of template DNA. Amplification was carried out in a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad, Hercules, USA). The PCR program consisted of a 10 min initial denaturation at 94°C, followed by 30 cycles of 40 s at 95°C, 45 s at 56°C, and 45 s at 72°C with a 10 min final elongation step at 72°C. PCR products were resolved on 1.2% agarose electrophoresis gel and visualized under UV light with ethidium bromide (Špitalská et al. 2002). DNA of *C. burnetii* (strain Nine Mile phase I, Institute of Virology, Slovak Academy of Science, Bratislava) was used as positive control for the amplification of *C. burnetii* DNA.

Results

During the first feeding experiment (Exp1), 514 larvae were fed successfully. Four out of five guinea pigs inoculated by *C. burnetii* became infected, as revealed by positive MAR reaction (titres ≥1:256) and positive ELISA reaction (titres ≥1:2,000). One *C. burnetii*-inoculated (P5) as well as the control guinea pig (K1) remained uninfected. Among 235 PCR-examined molted nymphs fed (as larvae) on four *C. burnetii*-positive guinea pigs, only 13 nymphs (5.6%) became infected. Notably, all 37 PCR-examined nymphs from one of the *C. burnetii*-positive guinea pigs (P3) remained PCR negative (Fig. 2).

In the second experiment, all guinea pigs exposed to *H. aegyptium* nymphs fed in Exp1 on *C. burnetii*-positive guinea pigs, became infected (MAR titres ≥1:256, ELISA titres ≥1:2,000). Importantly, DNA of *C. burnetii* was detected at 383 dpi in 26 out of 90 adult ticks (28.9%) that were fed at larval stage on *C. burnetii*-positive guinea pigs (Fig. 2).

All 14 nymphs fed on the control (K1) guinea pig and 45 nymphs fed on the uninfected P5 specimen remained PCR negative. Also, the guinea pigs exposed to nymphs fed previously on the control (K1) or P5 specimen remained uninfected. Similarly, all 130 adult ticks fed in larval stage on uninfected guinea pigs (and in Exp2 on guinea pigs K2, P11, and P12) were found to be *C. burnetii*-negative (Fig. 2).

Discussion

The study presented here proved the ability of the tortoise tick *H. aegyptium* to acquire *C. burnetii* infection from infected mammalian hosts. Moreover, the proven transstadial passage from larvae to nymphs and from nymphs to adults suggests the possibility of further transmission to mammals. Remarkably, adult ticks were found to be *C. burnetii*-positive 383 dpi, meaning that individual ticks can serve as long-term carriers, or reservoirs, of *C. burnetii*.

According to our results, the efficacy of *H. aegyptium* larvae to acquire infection when feeding on a *C. burnetii*-positive host is low. Notably, we detected higher prevalence of *C. burnetii* among adult ticks compared to the prevalence among nymphs. This trait could be caused by transmission of nonsystemic infection via co-feeding among closely aggregated feeding nymphs within feeding chambers (Randolph 1998; Randolph et al. 2002). Another explanation could be sought in the lower amount of template *C. burnetii* DNA obtained from smaller infected nymphs. Finally, there also could be coherence of this notable trait with collective keeping of each particular group of ticks as we cannot definitively exclude possible cross-contamination between ticks (e.g., via ticks' feces) (Hirai and To 1998).

Interestingly, all examined ticks fed in larval stage on the *Coxiella* serologically-positive P3 guinea pig were PCR negative. Nevertheless, some of six vial mates of these ticks transmitted *C. burnetii* infection to guinea pig P8 in Exp2, although all these six ticks were 291 days later examined by PCR with negative result. It is difficult to explain this phenomenon. We could hypothesize a decrease in intensity of *C. burnetii* infection in adult ticks over a long time period.

We did not test the exact route of *C. burnetii* transmission between ticks and guinea pigs. There exist different ways of transmission—via the tick saliva, by excretions of coxal glands, or by tick feces contamination (Řeháček and Brezina 1968). Transmission by the tick feces is known for example in the sheep tick *Dermacentor marginatus* (Hartelt et al. 2008; Hellenbrand et al. 2005). Since feeding of both larvae and nymphs of *H. aegyptium* was quite fast, we could only hypothesized, without any evidence, the other way than via feces contamination.

We are aware of no report dealing with natural *C. burnetii* infection of tortoises of the genus *Testudo*. We also have information about no study focused on identifying *C. burnetii* from naturally infected *H. aegyptium* collected from these tortoises. Nevertheless, according to the present study and with respect to studies of Blanc et al. (1959),

Blanc and Ascione (1960) and Blanc (1961), the potential of *Testudo* tortoises and their tick in the epidemiology of Q fever natural foci should not be overlooked.

Undoubtedly, reptiles can play an important role in maintaining natural foci of infectious diseases, either as reservoirs or as carriers of infected ticks. Reptilian ticks have been reported to be involved or suspected in spreading of some serious disease agents. Among these, the monitor lizard tick *Amblyomma exornatum* harbored natural infections of *C. burnetii* in Guinea-Bissau (Arthur 1962), while Kim et al. (1978) described an outbreak of Q fever in New York in persons removing the ticks *Amblyomma nuttalli* from ball pythons (*Python regius*) imported from Ghana. African ticks *Amblyomma sparsum* and *A. marmoreum* can spread *Ehrlichia ruminantium* between wildlife and domestic cattle (Burridge et al. 2000; Peter et al. 2000), and *Rickettsia honei*, the causative agent of Flinders Island spotted fever, was isolated from *Bothriocroton hydrosauri*, a tick of lizards, snakes, and turtles (Stenos et al. 2003). Recent studies have reported on findings of *Borrelia burgdorferi* s.l. in lacertid lizards and their ticks *Ixodes ricinus* collected in Slovakia and Hungary (Földvári et al. 2009; Majláthová et al. 2006). In this light, the importance of tortoises and their ticks in maintaining natural foci of Q-fever should not be undervalued.

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