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Short Communication

Rickettsia buchneri, symbiont of the deer tick *Ixodes scapularis*, can colonise the salivary glands of its host

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Abstract

Vertically-transmitted bacterial symbionts are widespread in ticks and have manifold impacts on the epidemiology of tick-borne diseases. For instance, they may provide essential nutrients to ticks, affect vector competence, induce immune responses in vertebrate hosts, or even evolve to become vertebrate pathogens. The deer or blacklegged tick *Ixodes scapularis* harbours the symbiont *Rickettsia buchneri* in its ovarian tissues. Here we show by molecular, proteomic and imaging methods that *R. buchneri* is also capable of colonising the salivary glands of wild *I. scapularis*. This finding has important implications for the diagnosis of rickettsial infections and for pathogen-symbiont interactions in this notorious vector of Lyme borreliosis.

Keywords

Symbiosis, rickettsiosis, Midichloria, biotin synthesis.

1. Introduction

The deer or blacklegged tick, *Ixodes scapularis*, is the most important vector of human tick-borne pathogens in the Eastern United States, playing a primary role in the transmission of the agents of Lyme borreliosis, babesiosis, human granulocytic anaplasmosis and Powassan encephalitis. However, in contrast with several other generalist species of *Ixodes* worldwide, *I. scapularis* is not a vector of spotted fever-group rickettsiae. Instead, *I. scapularis* harbours a rickettsial symbiont that is positioned in a clade distinct from the traditional spotted fever, typhus, ancestral and transitional groups, which were believed to encompass most rickettsial diversity until recently (Pilgrim et al., 2017). This "rickettsial endosymbiont of *I. scapularis*" or REIS (also known as "*Rickettsia* genotype Cooleyi") has been recognised for over two decades from microscopic and molecular studies and has never been associated with disease in vertebrates (Gillespie et al., 2012; Noda et al., 1997). Following screening of different lifecycle stages and individual organs from adult ticks from a laboratory colony, the symbiont was reported to be restricted to the ovaries of its host (Munderloh et al., 2005; Noda et al., 1997). However, very high-prevalence infections in the wild - including in adult males (Hagen et al., 2018; Moreno et al., 2006) - indicate that the symbiont must infect non-ovarian tissues, at least in certain populations. In 2012, the genome of REIS was published and shown to be significantly larger (>2 Mb) than that of pathogenic rickettsiae (Gillespie et al., 2012). It was also supplemented by an accessory genome consisting of four plasmids, one of which encodes complete operons for biotin biosynthesis (Gillespie et al., 2012). Subsequently, following isolation from *I. scapularis* ovaries and maintenance in a tick cell line, REIS was formally described as *Rickettsia buchneri* (Kurtti et al., 2015).

Several genera of bacteria (*e.g.*, *Rickettsia, Coxiella, Francisella, Midichloria* and *Rickettsiella*) are stably associated with different species of ticks, persisting through transstadial transfer

to be transmitted transovarially (Duron et al., 2017). These symbionts may remain strictly restricted to the arthropod host or can be transmitted to the ticks' vertebrate host, sometimes causing disease (Angelakis et al., 2016; Duron et al., 2017). A prerequisite for horizontal transmission of a symbiont to the vertebrate host (and potentially between ticks feeding on the same host) is colonisation of the tick's salivary glands. This process does not necessarily imply a transition to pathogenicity, being simply a transmission route to other ticks via co-feeding; nevertheless, a symbiont residing in this location will be exposed to the vertebrate host's immune system, typically leading to an antibody response. Moreover, the symbiont may interact with protozoal, bacterial and viral pathogens in the salivary glands, perhaps facilitating or impeding their transmission. Here, we demonstrate using a combination of molecular, proteomic and imaging methods that contrary to prior reports, *R. buchneri* colonises the salivary glands of *I. scapularis*.

2. Methods

Adult *Ixodes scapularis* ticks were collected from white-tailed deer (*Odocoileus virginianus*) by arrangement with the New Hampshire Fish and Game Department. Field scientists from this organisation removed ticks using fine forceps from deer shot by hunters within a 5-mile radius of a weigh station located near Manchester, NH. The specimens were frozen at -20°C and sent to New England Biolabs, where they were sorted based on degree of engorgement. Partially-fed ticks, estimated to have been attached to the host for 3 - 4 days (TickEncounter, 2018), were transferred into 70% ethanol and shipped to the University of Milan for dissection. Ticks were inspected thoroughly under a dissecting microscope to ensure they were not damaged. During and after the dissection, organs were checked microscopically for integrity and cross-contamination, and only intact tissues were kept for downstream

processing. Ten ovaries (OV) and 10 salivary gland pairs (SG) from individual ticks were pooled and preserved in ethanol. For proteomic analysis, the samples were lysed in 1% (w/v) sodium deoxycholate (Sigma) in 50 mM ammonium bicarbonate followed by three cycles of sonication on ice (Vibra-cell 130PB sonicator, 20 Hz, with microprobe; 10 s sonication alternating with 30 s incubation on ice). The samples were centrifuged at 13,000 × g for 10 min at 4°C to remove insoluble material and the pellets were used for DNA extraction in a Qiagen Blood and Tissue DNeasy kit according to the manufacturer's protocol. Soluble protein concentrations were measured using the Pierce Coomassie Plus (Bradford) protein assay (Bio-Rad, Hercules, CA, USA).

For geLC-MS, proteins were separated under denaturing conditions using 4 - 12% NuPAGE Bis-Tris protein gels (Invitrogen). Approximately 30 µg of the pooled OV and SG lysates were loaded onto the gel, and electrophoresis was conducted at 200 V (constant) for 35 min. The gel was stained overnight with GelCode Blue Stain Reagent (ThermoFisher Scientific). The entire gel lane was excised into seven equal slices (~1 mm wide), which were then cut into smaller pieces (~1 mm^3). Gel pieces were destained in 50% acetonitrile/50 mM ammonium bicarbonate (pH ~8), reduced for 30 min at 37°C with 10 mM dithiothreitol (Sigma) in 50 mM ammonium bicarbonate, and alkylated with 55 mM iodoacetamide (Sigma) in 50 mM ammonium bicarbonate for 30 min in the dark at room temperature. Gel pieces were washed for 15 min in 50 mM ammonium bicarbonate and dehydrated with absolute acetonitrile. Acetonitrile was removed, and the gel plugs rehydrated with 0.01 µg/µL proteomic grade trypsin (Sigma) in 50 mM ammonium bicarbonate. Digestion was performed overnight at 37°C. Peptides were extracted from the gel plugs using successive 15 min incubations in 3% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Peptide extracts were pooled and reduced

to dryness using a centrifugal evaporator (Eppendorf Concentrator Plus), then re-suspended in 3% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid for analysis by mass spectrometry (MS).

Peptides were analysed by liquid chromatography-MS/MS using a Q-Exactive mass spectrometer platform (Thermo Fisher Scientific). Each Thermo RAW file was imported into Progenesis QI (version 4.1, Nonlinear Dynamics) individually. Peak picking, instrument and Mascot parameters were as described elsewhere (Dong et al., 2018). Tandem MS data were searched against a combined database from *I. scapularis* (Uniprot, June 2017) and REIS (NCBI BioProject PRJNA33979); a total of 22,584 combined sequences and 6,279,968 residues. Separate sample fractions were combined using Progenesis QI to create one output file. Quantitative analysis for proteins detected in both ovaries OV and SG was performed in Progenesis QI for proteomics. Protein intensity values were used to create heat-maps using the Morpheus online tool (Morpheus, https://software.broadinstitute.org/morpheus).

Both conventional PCR and qPCR (TaqMan assay) targeting the *Rickettsia* spp. *gltA* gene (Stenos et al., 2005) were used on the OV and SG tissue extracts (one pool from 10 ticks per tissue). Starting copy numbers were quantified by linear regression in Opticon Monitor software v. 3.1. (Bio-Rad), using a dilution series of a synthetic standard (Eurofins Genomics) representing the 74-bp *gltA* amplicon. Normalisation of the qPCR against a tick single-copy nuclear gene, ribosomal protein L6 (*rpl6*), was done as described previously (Al-Khafaji et al., 2019). The conventional PCR used a previously published primer set and protocol (Roux et al., 1997), and PCR products from SG and OV were purified using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. Sequences were obtained by the Sanger method from Eurofins MWG Operon (Ebersberg, Germany), identified using BLAST (www.ncbi.nlm.nih.gov/BLAST), and compared with the reference gene *gltA* sequence

(WP_014409697.1; *R. buchneri* strain ISO7 contig076, whole genome shotgun sequence) available in GenBank. ClustalW was used for DNA sequence alignments.

Additional, partially-fed specimens of *I. scapularis* were collected live by co-authors CKSC and ZL from culled white-tailed deer in New Hampshire for fluorescence *in-situ* hybridisation. Ticks were kept at 4°C during transportation to the laboratory in Massachusetts and confirmed to be viable prior to dissection. The SG tissues were fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature, washed once, and transferred into 50% ethanol in PBS. The fixed SG were stored at -20°C prior to refrigerated shipment to the University of Milan. After thorough washing three times (5 min) with PBS-Tween 20 buffer [137 mM sodium chloride, 8.1 mM disodium phosphate, 2.7 mM potassium chloride, 1.5 mM monopotassium phosphate (pH 7.4), 0.2% Tween 20], the salivary glands were incubated with hybridization buffer [20 mM Tris-hydrochloride (pH 8.0), 0.9 M sodium chloride, 0.01% sodium dodecyl sulfate, 30% formamide] three times for 5 min at 42˚C. Then, the samples were hybridized with hybridization buffer containing the probes (100 nM each) at 42˚C overnight. A Cy3-labeled *Rickettsia*_B1-specific probe (5'-CCATCATCCCCTACTACA-3') specific for the genus *Rickettsia* (Perotti et al., 2006) and the Cy5-labeled probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3'), routinely used as a universal bacterial probe (Amann et al., 1990) were used in parallel. A non-*Rickettsia*_B1 probe, complementary to the *Rickettsia*_B1 probe, was used as a control. After thorough washing with PBS-Tween 20 (20 min), 75 ng/ml of DAPI was added for nuclear staining and incubated for 20 min at room temperature. The treated samples were mounted on slides with buffered glycerol containing 4% n-propyl gallate. The slides were observed using a laser-scanning Nikon A1R confocal microscope.

3. Results and Discussion

The qPCR assay detected *R. buchneri* DNA in both OV and SG of *I. scapularis* (Fig. 1). However, the bacterial density was approximately eleven-fold higher in OV (bacterial:tick genome ratio of 6.04 in OV and 0.55 in SG). Conventional PCR followed by Sanger sequencing detected *gltA* DNA that was 100% identical to that of *R. buchneri* in both tissues (alignment of 686 bp for OV and 530 bp for SG). Moreover, geLC-MS demonstrated the presence of *R. buchneri* proteins in both OV and SG. A comparison of expression levels of 29 proteins (high-confidence hits with ≥2 unique peptides) between the two tissues showed that most proteins could only be detected in OV; however, two chaperonins, heat-shock protein 20, and peptidoglycan-associated lipoprotein were robustly detected in SG (Fig. 2). Interestingly, several proteins encoded by the plasmids of *R. buchneri*, including a biotin synthase expressed in OV, were detected by the geLC-MS analysis (Fig. 2).

Of the six tick SG specimens examined, two showed clear evidence of rickettsial infection in the lumen by confocal microscopy. One specimen exhibited particularly extensive colonisation of the SG, with distinct clusters of bacteria accumulating in the cytoplasm of the acinar cells (Fig. 3).

Taken together, the evidence presented here demonstrates that *R. buchneri* can colonise the SG of *I. scapularis*. The most important implication of this finding is that vertebrate hosts of *I. scapularis*, including humans, could be exposed to *R. buchneri* antigens and possibly intact (potentially viable) bacteria. This raises interesting parallels with *Candidatus* (*Ca.*) Midichloria mitochondrii, the primary symbiont of *Ixodes ricinus* in Western Europe, which induces antibody responses in humans (Mariconti et al., 2012). Moreover, circulating symbiont DNA has been detected in the blood of various non-human hosts parasitized by *I. ricinus* (Bazzocchi et al., 2013), and phylogenetic analyses strongly suggest that horizontal transmission of *Ca*.

M. mitochondrii does occur at significant frequencies (Al-Khafaji et al., 2019; Buysse and Duron, 2018; Cafiso et al., 2016). Although this symbiont is not known to cause disease in vertebrates, there is interest in using the immune response against *Ca*. M. mitochondrii as a biomarker of tick bite in humans (Mariconti et al., 2012). If *R. buchneri* also generates antibody responses in humans, there is the potential for cross-reactivity in the serological tests used to diagnose the spotted-fever group infections transmitted by other ticks. This possibility deserves further attention, as immune responses to a symbiotic *Rickettsia* spp. could lead to unnecessary or inappropriate treatment of individuals being investigated for tick-borne diseases. Similar questions have arisen regarding the Q-fever agent, *Coxiella burnetii*, and *Coxiella*-like symbionts in ticks, which can also infect the SG of their hosts (Buysse et al., 2019; Duron et al., 2015; Klyachko et al., 2007).

It is unclear why prior studies have not reported the detection of *R. buchneri* in *I. scapularis* SG. However, very few tissue-specific analyses of *R. buchneri* distribution have been performed and the only published data are from laboratory colonies of *I. scapularis* (Noda et al., 1997), or from field-collected ticks that were subsequently reared in the laboratory (Munderloh et al., 2005). Moreover, most prior studies appear to have focused exclusively on unfed specimens. Considering our findings and previous observations *in toto*, it appears that the act of blood feeding may stimulate *R. buchneri* colonisation of the SG and/or this process of colonisation is not stimulated (or is somehow impeded) under laboratory conditions. Notably, a recent molecular study of *R. buchneri* using fed tick specimens collected by veterinary clinics reported a prevalence of 94% in adult females and 76% in males (Hagen et al., 2018). The highly sensitive digital-droplet PCR method applied in that study revealed a 2.2-fold greater symbiont density in females compared with males, which might reflect the presence of the bacteria in male SG (perhaps the only location colonised in this sex) versus

female OV and SG. It is also possible that *R. buchneri* exhibits similar behaviour to other symbionts, such as *Ca*. M. mitochondrii*,* in undergoing a "bloom" during tick engorgement (Sassera et al., 2008). Intriguingly, it was reported in the early 1990s that ~10% of field-collected *I. scapularis* harbour *Rickettsia*-like organisms in their haemocytes (Magnarelli et al., 1991). If these organisms were *R. buchneri*, the infection of haemocytes would provide a potential route for colonisation of the SG, as has been reported for *Anaplasma phagocytophilum* (Liu et al., 2011). However, release of *R. buchneri* cells from the OV that are then scavenged by haemocytes, leading to contamination of other tissues with symbiont products, cannot be discounted at this stage.

Rickettsia buchneri and its counterpart in *Dermacentor* spp. ticks, *Rickettsia peacockii*, exhibit gene inactivation (pseudogenisation) of various virulence determinants, such as variable outer membrane proteins, actin-based motility and some aspects of LPS biosynthesis (Gillespie et al., 2012). However, unlike many conventional bacterial pathogens, the pathogenicity of rickettsiae is a consequence of extensive genome reduction rather than the acquisition of virulence factors (Diop et al., 2019). Thus, it is possible that these symbionts may evolve the capacity to infect and induce disease in vertebrates, as has been demonstrated for *Coxiella*-like symbionts, in which rare cases of human illness caused by these normally tick-restricted organisms may mirror the emergence of the Q-fever agent, *Coxiella burnetii* (Angelakis et al., 2016). Indeed, recent studies across the gamut of vertically-transmitted tick symbionts support a paradigm in which dynamic shifts between arthropod-restricted symbiont and vertebrate pathogen oscillate back-and-forth within bacterial genera.

In common with the symbionts of other obligate haematophagous arthropods, it is now apparent that a major role for tick symbionts is to provision B vitamins that are deficient in

an exclusively sanguineous diet. For instance, in the soft tick *Ornithodoros moubata*, removal of a *Francisella*-like symbiont via antibiotic treatments leads to inhibition of moulting and death, which can be rescued by artificial B-vitamin supplementation (Duron et al., 2018). A similar role is suspected for *Coxiella*-like symbionts in *Rhipicephalus* spp. ticks (Gottlieb et al., 2015; Guizzo et al., 2017; Smith et al., 2015), and the presence of laterally-transferred biotin operons on a *R. buchneri* plasmid is suggestive of vitamin provisioning, as this pathway is entirely absent in spotted fever-group rickettsiae (Gillespie et al., 2012). This symbiont is also capable of *de novo* folate biosynthesis in common with many other *Rickettsia* spp. (Hunter et al., 2015). To the best of our knowledge, the proteomic detection of *R. buchneri* biotin synthase in *I. scapularis* OV is the first evidence that the biotin biosynthesis pathway is expressed *in situ*.

In conclusion, the colonisation of *I. scapularis* SG by its symbiont *R. buchneri* reported here is a finding with several ramifications for the diagnoses of tick-borne diseases and the emergence of new tick-borne pathogens. In addition, the localisation of *R. buchneri* in the tick SG indicates the potential for competitive interactions between the symbiont and tick-borne pathogens, as has been demonstrated previously for *Rickettsia* spp. in the ovaries of *Dermacentor andersoni* (Macaluso et al., 2002) and between *Borrelia burgdorferi* sensu stricto and the gut microbiota of *I. scapularis* (Narasimhan et al., 2014). Indeed, the incidence of co-infection between *R. buchneri* and *B. burgdorferi* in wild male *I. scapularis* has been reported to be lower than expected by chance (Steiner et al., 2008), an observation that deserves further research in light of our findings.

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Figure legends

Fig. 1. Detection of *R. buchneri gltA* in pooled salivary glands and ovaries of *I. scapularis.* A. Quantification of rickettsial *gltA* from SG (red stars) and OG (purple stars) in comparison with a standard curve of synthetic *gltA* amplicons (black circles). Samples were assayed in duplicate without dilution. B. Quantification of *I. scapularis rpl6* (for normalisation of rickettsial load) from SG (red stars) and OG (purple stars) in comparison with a standard curve of synthetic *rpl6* amplicons (black circles). Samples were assayed singly, both undiluted and at a 1/10 dilution (indicated on figure). Ct, cycle threshold.

Fig. 2. Heatmap representing relative intensity values for 29 *R. buchneri* proteins between salivary glands and ovaries of *I. scapularis*. Proteins shown were identified by ≥2 unique peptides and if encoded by a plasmid, this is indicated in parentheses.

Fig. 3. Fluorescence *in situ* hybridization of *R. buchneri* in *I. scapularis* salivary glands. A) Image obtained from light transmission. B) Overlay image of green signal from the *Rickettsia*_B1 probe and red signal from the EUB338 universal bacterial probe. Arrows indicate clusters of *Rickettsia* bacteria. C) Higher magnification (40× objective) image of overlay obtained with the *Rickettsia*_B1 (green) and EUB338 (red) probes. D) Salivary glands stained with non-*Rickettsia*_B1 probe (negative control) and EUB338 universal probe (red). Blue represents the nuclear counterstain (DAPI).

Fig 1

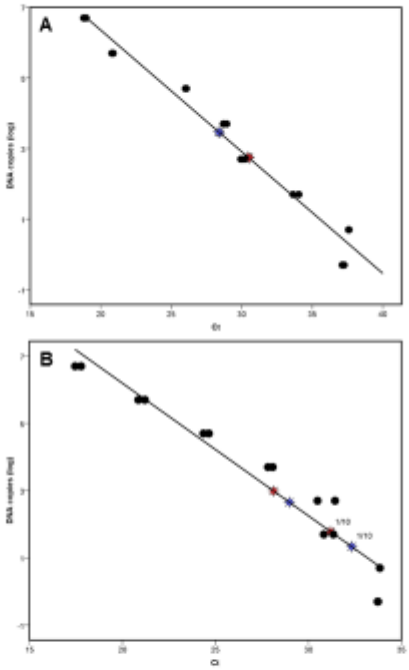

Fig 2

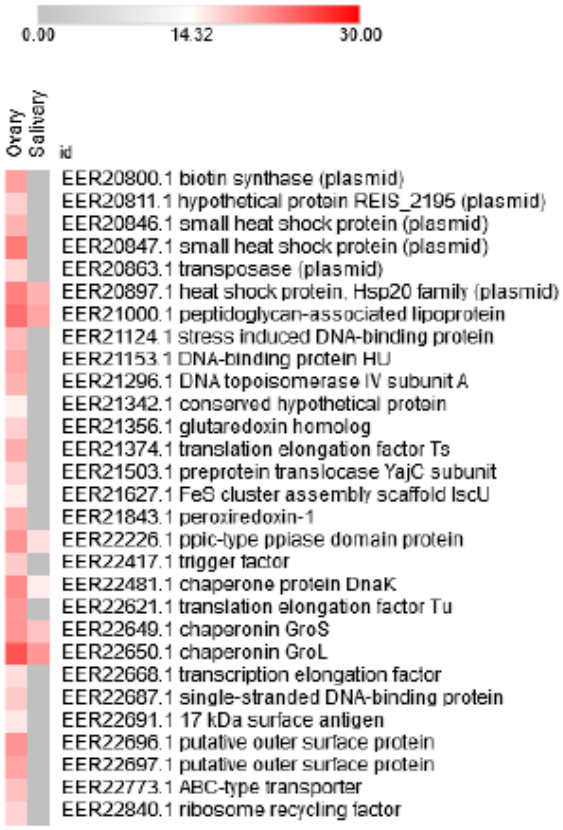

Fig 3