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Molecular Detection of *Rickettsia bellii*, *Rickettsia montanensis*, and *Rickettsia rickettsii* in a *Dermacentor variabilis* Tick from Nature

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Abstract

Rickettsial diseases, such as Rocky Mountain spotted fever, pose a public health threat because of humans' interrelationship with common arthropod species, such as ticks, mites, fleas, and lice. Individuals may come in contact with these vectors of disease on a fairly regular basis either directly or indirectly through pets or wildlife species, at home or in recreational areas. Therefore, it is of vital importance to know and understand the geographical distribution and prevalence of disease and rickettsial-infected arthropods. We analyzed *Dermacentor variabilis* ticks from nature found positive for *Rickettsia* sp. to determine the specific species present. Rickettsiae were detected through a 17-kDa surface antigen seminested PCR. Seminested PCR represents a sensitive and specific molecular technique in which to identify the presence of bacteria within arthropod hosts. Through sequence analysis of this gene, three *Rickettsia* species, *Rickettsia bellii*, *Rickettsia montanensis*, and *Rickettsia rickettsii*, were detected in a single tick specimen. Further molecular analyses of the 17-kDa surface antigen and the citrate synthase gene were also performed to support this finding. This is the first report of the detection of multiple *Rickettsia* species from a single *D. variabilis* tick in nature.

Key Words: PCR—*Rickettsia*—Tick(s)—Transmission—Vector-borne.

Introduction

Rickettsia is a genus of obligate intracellular bacteria associated with several human diseases. Arthropods, including ticks, fleas, and lice, are vectors for rickettsial disease as well as the primary hosts for these bacteria. Humans acquire rickettsial infections either by the bite of an infected tick or by the inadvertent rubbing of flea or louse feces into the skin or mucous membranes. In the United States, Rocky Mountain spotted fever (RMSF) is a potentially fatal tick-borne disease that is commonly found in every state, including Ohio. The etiological agent is *Rickettsia rickettsii* and in the southeastern states is transmitted primarily by the American dog tick, *Dermacentor variabilis*. Human cases of RMSF have occurred at a consistent rate over recent time in Ohio; from 1965 to 2007 the number of cases ranged from 10 to 36 per year with an average of 18 ± 7 (Ohio Department of Health RMSF fact sheet 2008). However, *R. rickettsii* is rarely found in *D. variabilis*, and the frequency of this bacteria is <1% (McDade and Newhouse 1986, Burgdorfer 1988, Ammerman et al. 2004, Perlman et al. 2006, Ohio Department of Health RMSF fact sheet 2008).

Most rickettsiae are maintained within their invertebrate hosts by means of transovarial and subsequent transstadial transmission. Additionally, rickettsiae can be acquired by horizontal transmission through the salivary glands of the arthropod host as it feeds on a vertebrate host in the wild, and as a result new lines of transovarial transmission can be initiated. Transovarial transmission is an extremely effective mechanism of rickettsial transmission, with transovarial and filial infection rates potentially reaching 100% (Burgdorfer 1963, 1988, Burgdorfer and Brinton 1975, McDade and Newhouse 1986, Perlman et al. 2006). Many nonpathogenic species, such as *Rickettsia bellii* and *Rickettsia montanensis*, have been shown to utilize transovarial and transstadial transmission in laboratory studies, and this is also most likely the case in nature (Macaluso et al. 2001, Horta et al. 2006). Further, *R. bellii* has been found at a high frequency, up to 80%, in ticks from nature, further suggesting transovarial transmission as the primary means of maintenance within the population of

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ticks (Philip 1983, Kelly et al. 2005, Horta et al. 2006). Pathogenic species, such as *R. rickettsii*, tend to have negative effects (host lethality) on their invertebrate hosts, effects that are thought to prevent the continued transovarial transmission of the pathogenic organism, and may explain why the prevalence is so low within arthropod hosts (Price 1953, Burgdorfer and Varma 1967, Burgdorfer and Brinton 1975, Burgdorfer 1988, Niebylski et al. 1999, Perlman et al. 2006).

Maintenance of multiple infections of *Rickettsia* sp. are believed to be prevented by ovarian interference, the resistance of the tick ovaries to infection with more than one rickettsial species; they have not been readily found in naturally occurring arthropods and have been demonstrated in few laboratory studies (Williams et al. 1992, Schriefer et al. 1994, Niebylski et al. 1997, Azad and Beard 1998, Noden et al. 1998, Stromdahl et al. 2001, Boostrom et al. 2002, Macaluso et al. 2002). Nonetheless, there is some evidence supporting the potential for arthropods to acquire a multiple rickettsial infection. Coinfection of *Rickettsia rhipicephali* and *R. bellii* has been reported in *Dermacentor occidentalis* ticks from California (Lane et al. 1981, Wickso et al. 2008). One laboratory study showed *R. rickettsii* could infect tissues of *Rickettsia peacockii*–infected ticks, where only the ovarian tissues remained infected exclusively with the east-side agent (*R. peacockii*) (Burgdorfer et al. 1981). Another study determined that in the laboratory *Rickettsia felis*–infected fleas could also acquire a *Rickettsia typhi* infection, but at a reduced rate relative to uninfected fleas (Noden et al. 1998). Finally, *Rickettsia* sp. have been found in coinfections alongside microbes of a distinct genus, which includes *Borrelia lonestari*, *Ehrlichia* sp., and a viral species (Gosteva et al. 1991, Mixson et al. 2006, Stromdahl et al. 2008). More sensitive screening assays might identify rickettsial multiple infections in nature. Each species might not occur in equal proportions within the host, and the minority species could have been missed during laboratory analysis or transmission studies. Conventional PCR, which uses only a single step, has been utilized in many of these tick screening analyses (Niebylski 1997, Stromdahl et al. 2001, Ammerman et al. 2004). Here, we employed the highly sensitive seminested PCR assay to test naturally infected *D. variabilis* ticks from Ohio. As a result, we have identified a single tick with the presence of *R. bellii*, *R. montanensis*, and *R. rickettsii*. This represents the first report of such a rickettsial mixed infection from *D. variabilis* in nature.

Materials and Methods

Samples

Fifty-six *D. variabilis* ticks were obtained from the Ohio Department of Health: Zoonotic Disease Program (ODH-ZDP). Ticks were collected during the 2003 annual screen for RMSF; tick hemolymph was analyzed by Gimenez staining and all samples that were found to be positive for *Rickettsia* sp. were further analyzed by direct fluorescent antibody testing. Tick #03-797, an unengorged adult female obtained from a human host, was determined by the ODH-ZDP to be positive for *R. rickettsii* through direct fluorescent antibody testing.

DNA extraction

Ticks were surface sterilized in 70% ethanol for 10 min, followed by two washes in distilled water (5 min, 1 min). The ticks were then ground in 180 μL of phosphate-buffered saline using a sterile mortar and pestle. DNA was extracted using the animal cell protocol of the DNeasy® Tissue Kit (Qiagen, Valencia, CA) and eluted in 100 μL Buffer AE followed by a secondary elution in 50 μL.

17-kDa gene PCR

Seminested PCR was performed under the following standard conditions. One microliter of DNA was used as template in an initial 10 μL reaction. A negative control contained 1 μL of sterile water in place of DNA template. The primers used were 17k-5′ 5′GCTTTACAAAATTCTAAAA ACCATATA (Stothard 1995) and 17kD-3′ 5′CTTGCCATT GTCCRTCAGGTTG. Amplification was done in a Whatman Biometra T-Gradient thermocycler (Biometra, Gottingen, Germany) using thermocycling conditions of 95°C for 5 min, 35 cycles of 95°C for 1 min, 53°C for 1 min, and 60°C for 1.5 min, followed by a final extension at 60°C for 15 min. Secondary amplification was done using 2 μL of a 1:10 dilution of the primary amplification in a 25 μL reaction. The negative control contained 2 μL of a 1:10 dilution of the primary negative control. Primers used were 17k-5′ and 17kD-3′nest 5′TCACGGCAATATTGACC, at an annealing temperature of 56°C. *Rickettsia conorii* strain VR141 Moroc. was initially used as a positive control; subsequently, previously identified *R. bellii*–positive ticks were used.

17-kDa multiplex

Primary PCR amplification was performed as previously described for the 17-kDa gene. Secondary amplification was done at a 68°C annealing temperature using primers 17k-5′, and 17kD-3′montB 5′GCCTATTACAACTGTTTGAGTGTAC and 17kD-3′bellii 5′ACTGCTACCACTTGGTGCAGC.

Citrate synthase gene PCR

PCR was performed as for the 17-kDa gene but at an extension temperature of 68°C and the use of TITANIUM™ Taq DNA polymerase (Clontech, Palo Alto, CA). Primary amplification was done at an annealing temperature of 54°C, and the primers used were CS1dvar 5′ATGACCAATGAAAA TAATAAT and CS1273r 5′CATAACCAGTGTAAAGCTG (Roux et al. 1997). The primers used in the secondary amplification were CS409d 5′CCTATGGCTATTATGCTTGC and either CS956rSFG 5′GAATATACTCAGAACTACCG or CS856rBellii 5′AAAGCGATGCAATACCAGTACTGAC. Thermocycling conditions were the same as the primary reaction but at a 56°C annealing temperature.

Amplimer purification

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and eluted in 30 μL of elution buffer. Band isolates from the multiplex PCR were placed on an approximately 0.5-inch square piece of nylon and spun at 14,000 g for 1 min to elute the DNA.

DNA sequencing

PCR products were directly sequenced with primers 17k-5′, 17k-5′seq 5′GGTTCTCAATTYGG, or Cs409d, as appropriate, using the Big Dye version 1.1 sequencing kit following man-

ufacturer's instructions (Applied Biosystems, Foster City, CA). The nucleotide sequence was viewed using the ABI 310 Genetic Analyzer (Applied Biosystems).

Vector cloning

One microliter of a 1:10 dilution of the purified 17 kDa and citrate synthase PCR products was used as template in the TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA). All tentatively positive colonies were purified by QIAprep® Spin Miniprep Kit (Qiagen) and confirmed by an *Eco*R I (Invitrogen) restriction digest. Positive clones were sequenced using the M13 forward primer (Invitrogen) following sequencing methods described above.

Results

The 17-kDa surface antigen gene was analyzed from rickettsial-positive *D. variabilis* ticks obtained from the ODH-ZDP RMSF screen to identify the specific rickettsial species. The 17-kDa gene was selected because it is specific to the genus *Rickettsia* and has sufficient divergence to provide species-specific nucleotide sequences (Anderson 1990). The 17-kDa gene has been used for phylogenetic analysis of *Rickettsia* and is an excellent target for use in screening for the presence of rickettsial species in arthropod hosts because of its small size as a PCR target; it also has also been shown to have a higher prevalence of nucleotide substitutions than the 16S rRNA gene (Azad et al. 1992, Stothard 1995, Stenas 1998). Tick #03-797, an unengorged adult female from Hocking County, was found positive by seminested PCR, and the appropriate negative control yielded no detectable product. Upon analysis, the nucleotide sequence from the secondary PCR amplification of this tick suggested the presence of multiple amplimers. Dual electropherogram peaks were observed at numerous base positions throughout the nucleotide sequence. Further analysis revealed that the superimposed sequences were consistent with a combination of the known *R. bellii* sequence and the known *R. montanensis* sequence. Although not conclusive, results were consistent with the additional presence of the sequence for *R. rickettsii*. To confirm the presence of multiple species, the 17-kDa gene product was vector cloned and individual colonies were subsequently sequenced to identify all the rickettsial nucleotide sequences present. A set of six clones was found to contain species-specific sequence inserts corresponding to the 17-kDa gene sequence. Three clones were identical over a 386 bp region to *R. bellii* (Genbank accession NC_009883), two other clones were identical over this same region to the sequence of *R. montanensis* (Genbank accession RMU11017), and one clone showed one base difference from the *R. rickettsii* sequence (Genbank accession NC_009882) but shared six unique changes with *R. rickettsii* that differentiate that species from the sequence of *R. montanensis*. To further verify these results, the entire procedure, starting from the primary PCR amplification of the 17-kDa gene, was repeated. Subsequent sequence analysis of the 17-kDa gene product showed multiple electropherogram peaks, consistent with previous results. Individual clones of the PCR product from the second amplification were found with sequence inserts identical to *R. bellii* (4/12), *R. montanensis* (7/12), or differed by one base from *R. rickettsii* (1/12), but as before shared six nucleotide changes with *R. rickettsii* that distinguish that species from *R. montanensis*.

Based on the results of the cloning experiment, we chose to design alternative PCR primers that would permit the testing of samples in a multiplex PCR. These primers were focused within the 3′ end of the gene sequence and designed to be internal of the existing 17kD-3′ primer and to produce alternative PCR products of two different sizes. One primer was specific for *R. bellii*, while the second was specific to *R. rickettsii* and *R. montanensis*. Three effective species-specific primers could not be designed due to the close sequence similarity between *R. montanensis* and *R. rickettsii* (98.6%) and the location of the targeted sequence differences to the existing PCR primers. Multiplex PCR resulted in the amplification of two products of the predicted sizes for each primer set. The individual bands were isolated and sequenced using primers 17k-5, 17kD-3′bellii, or 17kD-3′montB. The *R. bellii*–specific amplimer was found to have a nucleotide sequence consistent with *R. bellii*. The *R. montanensis*/*R. rickettsii*–specific product resulted in a mixed nucleotide sequence. The mixed product was cloned, resulting in two positive clones. The clones were subsequently sequenced, and one was found to contain *R. montanensis*, and the other a *R. rickettsii*–specific insert.

We additionally analyzed the citrate synthase gene sequence to corroborate the presence of multiple rickettsial species. Here, primers were designed for analysis in two separate seminested reactions, one to amplify the *R. bellii* product and one to amplify the *R. montanensis*/*R. rickettsii* product. Separate primer sets were required due to the *R. bellii* sequence being sufficiently divergent from *R. montanensis* and *R. rickettsii* such that a universal primer set was not successful in amplifying all three agents. Positive results were obtained from both primer sets, and the appropriate negative control yielded no detectable product. The nucleotide sequences from these PCR products were consistent with *R. montanensis* and *R. bellii*. The *R. montanensis* product was cloned to determine if *R. rickettsii* was present in small amounts, but no *R. rickettsii*–specific clones were found (0/9).

Discussion

This is the first report of a natural superinfection of an arthropod with three different species of *Rickettsia*: *R. bellii*, *R. montanensis*, and *R. rickettsii.* Seminested PCR represents a highly sensitive assay for detection of microorganisms of interest present in an arthropod host such as *D. variabilis*. It is specific in amplifying a single gene, and subsequent sequence analysis can determine the corresponding nucleotide sequences. The combination of seminested PCR analysis of two independent genes followed by cloning and sequencing provides strong support for our conclusions of the presence of at least two and possibly three different rickettsial agents. The occurrence of multiple rickettsiae in a single tick is important for further understanding rickettsial transmission, RMSF ecology, and the potential for human acquisition of several rickettsial species.

The three rickettsial species considered here are all found in ticks from Ohio. *R. bellii* and *R. montanensis*, organisms considered to be nonpathogenic for mammals, are found at frequencies up to 80% of ticks examined (Price 1953, Linnemann et al. 1980, Gordon et al. 1984, Pretzman et al. 1990, Raoult and Roux 1997). The additional presence of *R. rickettsii* is more surprising since it is normally found at a very low frequency (McDade and Newhouse 1986, Burgdorfer 1988, Hackstadt

1996, Ammerman et al. 2004, Perlman et al. 2006, Ohio Department of Health RMSF fact sheet 2008). The analysis of the 17-kDa gene revealed a low concentration of *R. rickettsii* (1/9 and 1/12) within the mix of rickettsial DNA from our tick. This low frequency within the bacterial mix and the presence of the two additional rickettsial species may explain why *R. rickettsii* was not detected in the analysis of the citrate synthase gene. Replication may have been reduced depending on what and how many tissues were infected by *R. rickettsii*.

Tick #03-797 was an unengorged adult female, meaning any rickettsiae present had been previously acquired, either transovarially or through a bloodmeal, and maintained through one or two molts. One of the nonpathogenic species, *R. bellii* or *R. montanensis*, was most likely acquired transovarially, and due to ovarian interference, the alternate species and the pathogenic *R. rickettsii* were probably acquired from a feeding event. Perhaps, the acquisition of *R. rickettsii* occurred simultaneously with either *R. montanensis* or *R. bellii*, thus preventing it from establishing a stable infection in the tick. There is evidence that competition between rickettsiae favors a single nonpathogenic rickettsial infection (Macaluso et al. 2002), but this competition as well as competition with other bacterial species has not been well studied within tick hosts. Also, the initial amount of bacteria ingested by a tick during feeding is dependent on the stage of rickettsemia in the infected vertebrate (McDade and Newhouse 1986). If the tick fed while this animal was at the final stage, then the amount ingested may not have been sufficient. The above situations may explain why *R. rickettsii* was present only in low quantities in the 17-kDa analysis and absent in the citrate synthase analysis. These results are mirrored by the lack of correlation between the low prevalence of *R. rickettsii* observed in ticks compared to the geographical areas where RMSF is prevalent (Pretzman et al. 1990, Azad and Beard 1998, Raoult and Paddock 2005).

The possibility of contamination exists but seems remote given that all appropriate precautions were taken for sterile techniques, and negative controls were performed. In addition, analyses of other ticks, done in concert with the tick reported here, did not show any occurrences of multiple species. This included the 56 *D. variabilis* ticks analyzed here, an additional 33 *D. variabilis* and over 200 *Amblyomma americanum* tick pools and individual specimens analyzed as part of a larger project in the Fuerst laboratory. Further, *R. rickettsii* DNA had not been previously used in the laboratory, and could not explain the presence of *R. rickettsii* in this tick sample. Thus, this apparent multiple rickettsial infection appears to be an unusual isolated case.

Superinfection is common with many other bacterial species in nature. For example, insects are commonly infected with several symbionts, and different viral and/or bacterial species can infect the same host (Fukatsu et al. 2000, Sandstrom et al. 2001, Stromdahl et al. 2001, Gosteva et al. 1991, Skotarczak et al. 2002, Stanczak et al. 2002). In addition, there exist numerous consequences of a superinfected arthropod, including evolution of pathogen virulence, complications with diagnosis, and a synergistic influence in the vertebrate host including immune repression (Lipstitch et al. 1996, Sexton et al. 1998, Perlman et al. 2006). Since survival of some rickettsiae depends on frequent or occasional horizontal transmission events (i.e., *R. prowazekii*, the agent of epidemic typhus), multiply-infected arthropods may serve a role in nature for the maintenance of more virulent species. With the use of more sensitive molecular tools as utilized here, we may find more cases of superinfection, adding significantly to the understanding of the maintenance of these pathogens in nature.

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Disclosure Statement

No competing financial interests exist.

References

Ammerman, NC, Swanson, KI, Anderson, JM, Norris, DE. Spotted-fever group *Rickettsia* in *Dermacentor variabilis*, Maryland. Emerg Infect Dis 2004; 10:1478–1481.

Anderson, BE. The 17-kilodalton protein antigens of spotted fever and typhus group *Rickettsiae*. Ann NY Acad Sci 1990; 590:326–333.

Azad, AF, Sacci, JB, Nelson, WM, Dasch, GA, et al. Genetic characterization and transovarial transmission of a typhus-like *Rickettsia* found in cat fleas. Proc Natl Acad Sci USA 1992; 89:43–46.

Azad, AF, Beard, CB. Rickettsial pathogens and their arthropod vectors. Emerg Infect Dis 1998; 4:179–186.

Boostrom, A, Beier, MS, Macaluso, JA, Macaluso, KR, et al. Geographic association of *Rickettsia felis*-infected opossums with human murine typhus, Texas. Emerg Infect Dis 2002; 8:549–554.

Burgdorfer, W. Investigation of "Transovarial transmission" of *Rickettsia rickettsii* in the wood tick, *Dermacentor andersoni*. Exp Parasitol 1963; 14:152–159.

Burgdorfer, W. Ecological and epidemiological considerations of Rocky Mountain spotted fever and scrub typhus. In: Walker, DH, ed. *Biology of Rickettsial Diseases*, vol. 1. Boca Raton, FL: CRC Press Inc., 1988:34–50.

Burgdorfer, W, Varma, MGR. Trans-stadial and transovarial development of disease agents in arthropods. Annu Rev Entomol 1967; 12:347–376.

Burgdorfer, W, Brinton, LP. Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. Ann NY Acad Sci 1975; 90:770–781.

Burgdorfer, W, Hayes, SF, Mavros, AJ. Non-pathogenic rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsii*. In: Burgdorfer, W, Anacker, RL, eds. *Rickettsiae and Rickettsial Diseases*. New York: Academic Press, 1981:585–594.

Fukatsu, T, Nikoh, N, Kawai, R, Koga, R. The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). Appl Environ Microbiol 2000; 66:2748–2758.

Gordon, JC, Gordon, SW, Peterson, E, Philip, RN. Epidemiology of Rocky Mountain spotted fever in Ohio, 1981: serologic evaluation of canines and rickettsial isolation from ticks associated with human case exposure sites. Am J Trop Med Hyg 1984; 33:1026–1031.

Gosteva, VV, Klitsunova, NV, Rehacek, J, Kocianova, E, et al. Mixed rickettsia-virus infection in *Dermacentor reticulatus* imago. Acta Virol 1991; 35:174–186.

Hackstadt, T. The biology of Rickettsiae. Infect Agents Dis 1996; 5:127–143.

Horta, MC, Pinter, A, Schumaker, TSS, Labruna, MB. Natural infection, transovarial transmission, and transstadial survival of *Rickettsia bellii* in the tick *Ixodes loricatus* (Acari: Ixodidae) from Brazil. Ann NY Acad Sci 2006; 1078:285–290.

Kelly, DJ, Carmichael, JR, Booton, GC, Poetter, KF, et al. Novel spotted fever group Rickettsiae (SFGR) infecting *Amblyomma americanum* ticks in Ohio, USA. Ann NY Acad Sci 2005; 1063:352–355.

Lane, RS, Philip, RN, Casper, EA. Ecology of tick-borne agents in California: II. Further observations on rickettsiae. In Burgdorfer, W, Anacker, RL, eds. *Rickettsiae and Rickettsial Diseases*. New York: Academic Press, 1981:575–584.

Linnemann, CCJ, Schaeffer, AE, Burgdorfer, W, Hutchinson, L, et al. Rocky Mountain spotted fever in Clermont County, Ohio. II. Distribution of population and infected ticks in an endemic area. Am J Epidemiol 1980; 111:31–36.

Lipsitch, M, Siller, S, Nowak, MA. The evolution of virulence in pathogens with vertical and horizontal transmission. Evolution 1996; 50:1729–1741.

Macaluso, KR, Sonenshine, DE, Ceraul, SM, Azad, AF. Infection and transovarial transmission of *Rickettsiae* in *Dermacentor variabilis* ticks acquired by artificial feeding. Vector Borne Zoonot Dis 2001; 1:45–53.

Macaluso, KR, Sonenshine, DE, Ceraul, SM, Azad, AF. Rickettsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. J Med Entomol 2002; 39:809–813.

McDade, JE, Newhouse, VF. Natural history of *Rickettsia rickettsii*. Annu Rev Microbiol 1986; 40:287–309.

Mixson, TR, Campbell, SR, Gill, JS, Ginsberg, HS, et al. Prevalence of *Ehrlichia*, *Borrelia*, and *Rickettsial* agents in *Amblyomma americanum* (Acari: Ixodidae) collected from nine states. J Med Entomol 2006; 43:1261–1268.

Niebylski, ML, Schrumph, ME, Burgdorfer, W, Fischer, ER, et al. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. Int J Syst Bacteriol 1997; 47:446–452.

Niebylski, ML, Peacock, MG, Schwan, TG. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). Appl Environ Microbiol 1999; 65:773–778.

Noden, BH, Radulovic, S, Higgins, JA, Azad, AF. Molecular identification of *Rickettsia typhi* and *R. felis* in co-infected *Ctenocephalides felis* (Siphonaptera: Pulicidae). J Med Entomol 1998; 35:410–414.

Ohio Department of Health RMSF fact sheet. 2008. Available at http://www.odh.ohio.gov/odhPrograms/idc/zoodis/vbdp/vbtick.aspx.

Perlman, SJ, Hunter, MS, Zchori-Fein, E. The emerging diversity of *Rickettsia*. Proc R Soc B 2006; 273:2097–2106.

Philip, RN, Casper, EA, Anacker, RL, Cory, J, et al. *Rickettsia bellii* sp. nov.: A tick-borne *Rickettsia,* widely distributed in the United States, that is distinct from the spotted fever and tyhus biogroups. Int J Syst Bact 1983; 33:94–106.

Pretzman, C, Daugherty, N, Poetter, K, Ralph, D. The distribution and dynamics of *Rickettsia* in the tick population of Ohio. Ann NY Acad Sci 1990; 590:227–236.

Price, WH. Interference phenomenon in animal infections with Rickettsiae of Rocky Mountain spotted fever. Proc Soc Exp Biol Med 1953; 82:180–184.

Raoult, D, Roux, V. *Rickettsia* as paradigms of new or emerging infectious diseases. Clin Microbiol Rev 1997; 10:694–719.

Raoult, D, Paddock, CD. *Rickettsia parkeri* infection and other spotted fevers in the United States. New Engl J Med 2005; 353:626–627.

Roux, V, Rydkina, E, Eremeeva, M, Raoult, D. Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. Int J Syst Bacteriol 1997; 47:252–261.

Sandstrom, JP, Russell, JA, White, JP, Moran, NA. Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Ecol 2001; 10:217–228.

Schriefer, ME, Sacci, JBJ, Taylor, JP, Higgins, JA, et al. Murine typhus: updated roles of multiple urban components and a second typhuslike *Rickettsia*. J Med Entomol 1994; 31:681–685.

Sexton, DJ, Corey, GR, Carpenter, C, Kong, LQ, et al. Dual infection with *Ehrlichia chaffeensis* and a spotted fever group *Rickettsia*: a case report. Emerg Infect Dis 1998; 4:311–316.

Skotarczak, B, Wodecka, B, Cichocka, A. Coexistence DNA of *Borrelia burgdorferi sensu lato* and *Babesia microti* in *Ixodes ricinus* ticks for north-western Poland. Ann Agric Environ Med 2002; 9:25–28.

Stanczak, J, Racewicz, M, Kruminis-Lozowska, W, Kubica-Biernat, B. Coinfection of *Ixodes ricinus* (Acari: Ixodidae) in northern Poland with the agents of Lyme borreliosis (LB) and human granulocytic ehrlichiosis (HGE). Int J Med Microbiol 2002; 291:198–201.

Stenos, J, Roux, V, Walker, D, Raoult, D. *Rickettsia honei* sp. nov. the aetiological agent of Flinders Island spotted fever in Australia. Int J Sys Bact 1998; 48:1399–1404.

Stothard, D. *The Evolutionary History of the Genus* Rickettsia *as Inferred from 16S and 23S Ribosomal RNA Genes and the 17 kilodalton Cell Surface Antigen Gene, in Department of Molecular Genetics*. Columbus, OH: The Ohio State University, 1995:214.

Stromdahl, EY, Evan, SR, O'Brien, JJ, Gutierrez, AG. Prevalence of infection in ticks submitted to the human tick test kit program of the U.S. Army Center for Health Promotion and Preventive Medicine. J Med Entomol 2001; 38:67–74.

Stromdahl, EY, Vince, MA, Billingsley, PM, Dobbs, NA, et al. *Rickettsia amblyommii* infecting *Amblyomma americanum* larvae. Vector Borne Zoonot Dis 2008; 8:15–24.

Wickso, ME, Hu, R, Dasch, GA, Krueger, L, et al. Detection and identification of spotted fever group rickettsiae in *Dermacentor* species from Southern California. J Med Entomol 2008; 45:509–516.

Williams, SG, Sacci, JBJ, Schriefer, ME, Anderson, EM, et al. Typhus and typhuslike rickettsiae associated with opossums and their fleas in Los Angeles county, California. J Clin Microbiol 1992; 30:1758–1762.

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