THE TICK MICROBIOME: DIVERSITY, DISTRIBUTION AND INFLUENCE OF THE INTERNAL MICROBIAL COMMUNITY FOR A BLOOD-FEEDING DISEASE VECTOR

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ABSTRACT

Ticks are well established as important vectors for human disease, accounting for a growing number of zoonotic infections. Certain primary pathogens such as the Lyme disease agent, have received great attention. Less well understood is the overall microbial community that is harbored within ticks, in addition to human pathogens. A variety of powerful molecular detection approaches have revealed a constrained but significant microbial community associated with ticks, including vertically-transmitted symbionts, opportunistic pathogens, and more transient guest commensals, which include viruses, bacteria, protozoans, and fungi. Ticks join a growing number of arthropod and filarial systems in which microbial symbionts can have profound and extensive effects on the activity of their host and in certain cases, a direct impact on human disease. The recognized human pathogens are in fact vastly outnumbered by these other microorganisms, and pathogens represent a relatively small fraction of the total microbial community in ticks. The tick-borne microbial community affords the opportunity for functional interactions between microorganisms, which can have significant influence on the relative population sizes of the different resident microbial taxa. In ticks, limited evidence suggests that specific microbes or the overall microbial community can influence the acquisition, transmission and virulence of known pathogens such as *Borrelia burgdorferi, Anaplasma phagocytophilum*, or *Babesia microti*, as well as newly emerging pathogens. This area remains understudied at this point and represents a current gap in our knowledge. Future research efforts are required in light of recent results from other arthropod systems such as aphids and *Drosophila*, and will greatly benefit from new technologies for in-depth profiling of the tick microbiome, allowing high sampling depth for ecological investigations and for experimental laboratory approaches.

INTRODUCTION

Ticks (Class Arachnida, Order Acari) are blood-feeding arthropods that feed on terrestrial vertebrates and vector a diverse group of human and wildlife pathogens, including viral, bacterial, and protozoan disease agents (Sonenshine and Mather 1994; Goodman, Dennis, and Sonenshine 2005). Ticks vector more human pathogens than any other arthropod, and are the primary source of vector-borne infectious disease in many temperate areas (Asia, Europe, North America). Unlike other blood-feeding arthropods such as mosquitos, fleas and lice, ticks exhibit extended time periods between blood meals of up to a year or more. Ticks can acquire pathogens during blood meals but transmission of pathogens to susceptible vertebrate hosts depends on

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ticks maintaining their infections during transstadial molts (from larvae to nymphs and from nymphs to adults, (Sonenshine 1991). Most hard ticks (Ixodidae) have a three-stage life cycle (larvae, nymph, adult) and each blood meal may be from a different host species. As a result, pathogens are potentially spread widely among vertebrate species, making ticks important sources of zoonotic disease.

Ticks have been well-studied because of their human health impacts but new microbial associations continue to be described (Jasinskas, Zhong, and Barbour 2007; Grindle et al. 2003; Morimoto, Kurtti, and Noda 2006) and new emerging diseases are being recognized (e.g. Paddock and Yabsley 2007, STARI, Loftis et al. 2008, Panola Mountain *Ehrlichia*, LaSala and Holbrook 2010, viral haemorrhagic fevers). In addition to pathogens, ticks serve as hosts for a variety of endosymbiotic, vertically-transmitted bacteria, including *Coxiella*-, *Francisella*- and *Rickettsia*-like organisms (Perotti et al. 2006; Noda, Munderloh, and Kurtti 1997; Sun et al. 2000; Morimoto, Kurtti, and Noda 2006), and newly described symbionts of tick mitochondria (Sassera et al. 2006; Epis et al. 2008). Tick endosymbionts are often closely related to virulent human pathogens (Fig. 1). It is likely that ticks are host to a larger diversity of, as yet undiscovered, microbes.

Changing environmental conditions, including climate change, land-use patterns, wildlife populations and agricultural practices, are acting to alter host and tick ecology and their geographical distributions, leading to new regions of tick activity, overlapping distributions and emerging disease (Childs and Paddock 2003; McDiarmid et al. 2000; Masuzawa et al. 2008; Sun et al. 2008; van Overbeek et al. 2008; Randolph 2010). These dynamic changes are providing new opportunities for pathogen host shifts and mixed infections, including new microbial community associations (Eisen, Meyer, and Eisen 2007; Eisen 2008; Randolph and Rogers 2010).

Methodologies for Identifying and Enumerating Microbes in Ticks

The field of microbiology has relied for over a century on the ability to cultivate microorganisms derived from natural environments. Although this approach remains one of the most commonly employed and useful means of identifying microbes, it excludes the detection of a potentially vast range of microorganisms. Estimates from soil environments suggest that greater than 99% of active microorganisms are not detectable by conventional cultivation methods (Rondon et al. 2000; Hugenholz, Goebel, and Pace 1998). It is particularly clear that ticks and other arthropods frequently harbor microbes that have obligate intracellular life histories, either as commensals or pathogens, or are very difficult to cultivate (Dale and Moran 2006). Although traditional microscopy and histological staining can provide presumptive identifications of tick-associated microbes, the information is often ambiguous and of limited utility.

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FIGURE 1 Phylogram of representative a subset of 16s-rDNA phylotypes from ticks. Brackets indicate genera. Underlined entries from recent tick isolates. Dv, *D. variabilis*; Aa, *A. americanum*; Is, *I. scapularis*. Numbers at nodes are boot strap support. Source: Clay and Fuqua, unpublished

Powerful molecular approaches now enable the detection of microorganisms independent of the limitations of cultivation. Microbes can be identified and phylogenetically characterized, often to the level of genus and species, using nucleic acid or antibody probes directed towards highly conserved macromolecules (Clements and Bullivant 1991). Although a range of conserved proteins, fatty acids and nucleic acids have been used as targets for this purpose, small subunit ribosomal RNAs (SSUs) such as bacterial 16S rRNA, are the most generally applied, and often the most informative (Stahl 1995). No area of investigation has benefited more from these approaches than arthropod-microbe interactions. Molecular analyses have revealed diverse arthropod-associated microbes for a variety of systems (Dale and Moran 2006). Many of these microbes have not yet been cultured and their identification would be virtually impossible without cultivation-independent methods. Likewise, the study of tick-borne pathogens increasingly relies on molecular detection approaches to identify, distinguish and compare pathogens among different tick species and populations (Sun et al. 2000; Schabereiter-Gurtner, Lubitz, and Rölleke 2003; Burkot et al. 2001).

A number of studies have utilized Polymerase Chain Reaction (PCR) to amplify conserved microbial sequences, such as 16S rRNA gene sequences from total DNA extracts isolated from ticks, either as individuals or in small pools (Heise, Elshahed, and Little 2010; Clay et al. 2008; Benson et al. 2004). In the gene library sequence approach, these amplicons are ligated *en masse* into standard PCR cloning vectors, transformed into a cloning host such as *Escherichia coli*, and the plasmids are isolated from the initial transformants. The 16S rRNA amplicons carried on these plasmids are then sequenced and the source microorganism is deduced by comparison with rRNA gene sequence databases, such as the Ribosomal Database Project (http://rdp.cme.msu.edu/). This cultivation-independent approach has been tremendously informative, and has revealed a number of tick-associated microbes that would have never been identified otherwise (Adar, Simaan, and Ulitzur 1992; Grindle et al. 2003; Heise, Elshahed, and Little 2010; Jasinskas, Zhong, and Barbour 2007; Schabereiter-Gurtner, Lubitz, and Rölleke 2003). A major limitation is however numerical – each clone must be sequenced individually. Hundreds of plasmids may be generated from a single tick, and sequencing to significant depth per tick is extremely expensive and time consuming. In depth analysis of large numbers of individual ticks becomes prohibitive. This problem is exacerbated by the trend for there to be a single, highly abundant type of microbe that colonizes each tick to high density, and therefore a large fraction the 16 S rRNA gene sequences determined are from this one taxon.

Molecular community fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (T-RFLP) analysis, ostensibly provide efficient snapshots of microbial community composition (Muyzer and Smalla 1998; Osborn, Moore, and Timmis 2000). These approaches also utilize PCR to amplify diagnostic sequences from samples, again most typically 16S rRNA genes. The underlying microbial diversity in any given community is revealed by electrophoretic separation of amplicons with different sequences, providing a microbial fingerprint. Specific microbial identification is also possible with both DGGE and T-RFLP approaches, but in practice this is considerably less reliable than the library sequencing approach for microbial identification. These techniques have proven useful in analyzing certain microbial communities, but have only been employed sparingly to analyze tick microbiota (Schabereiter-Gurtner, Lubitz, and Rölleke 2003). They provide somewhat course resolution on microbial diversity, revealing the major trends in composition, and therefore the numerical dominance of a single symbiont taxon cited above also creates problems for this approach.

The molecular approaches described above provide a way to gauge diversity in a microbial community, but are not an efficient way to determine the presence or absence of specific microbes in a sample. Nor do they provide robust information on relative abundances of a given microbe. Once specific microbial taxa of interest have been identified, these microbes can be targeted directly. Direct and nested PCR based assays with primer sets specific to diagnostic sequences (often, but not always 16S rRNA genes) for targeted microbial groups allows highly sensitive detection (Clay et al. 2008). Fluorescent in situ hybridization (FISH) analysis of sectioned ticks with specific oligonucleotide probes allows visualization of the site(s) of colonization for specific microbial taxa (Fig. 2; Klyachko et al. 2007; Hammer et al. 2001). These taxon-specific assays are able to detect microbes at very low relative abundance that often escape the more general community approaches. Furthermore, quantitative PCR allows the relative abundance of specific microbes to be determined (Jasinskas, Zhong, and Barbour 2007). The directed approaches however also suffer from several limitations and complexities. Investigators must know precisely which microbe they aim to detect, and they must have high

confidence that the specific PCR primer set they employ is not confounded by cross-amplification of other microbes. Even minor divergence in the targeted sequences can lead to loss of detection, and it is difficult to trust PCR failures of a single target sequence as evidence for the absence of specific pathogens or other symbionts. Multilocus sequence typing (MLST) approaches target multiple conserved genes (usually 5-10) in a targeted microbe, again using PCR, but following this by sequencing of the amplicons (Maiden et al. 1998). Specific microbial lineages, or sequence types, are defined by the complete set of sequences obtained. This technique generally relies on the physical isolation of the targeted microbe from the sample however, most typically by cultivation, and has been used only to analyze closely related tick microbiota (Margos et al. 2008).

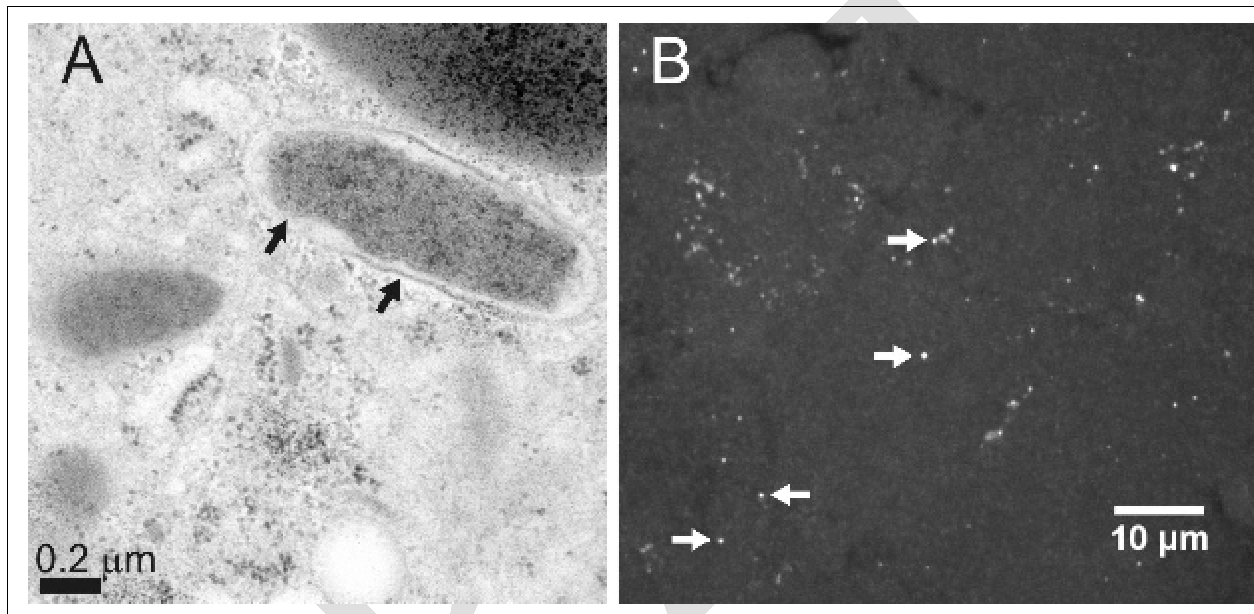

FIGURE 2 Visualization of *Coxiella*-type symbiont from *A. americanum*. (A) Transmission electron microscopy of thin sectioned ovarian tissue from a engorged female. Arrows demarcate presumptive outer and inner membrane structures (B) FISH microscopy of tissue section from a dissected ovary*A. americanum* show oocytes stained with a *Coxiella*-specific probe labeled with Cy5. Arrows highlight fluorescent punta resulting from probing and indicate of the *Coxiella*-type symbiont. Source: Klyachko et al. 2007.

The advent of high-throughput, next generation sequencing techniques promises to surmount many of the limitations in current molecular approaches to microbial diversity studies described above. The ability to obtain hundreds of thousands of individual sequences in a single run provides tremendous power to probe the depth and breadth of a wide range of microbial communities. In the analysis of microbial communities, short segments of the 16S rRNA gene are PCR amplified using specific primer sets to generate 16S "tags" (Sogin et al. 2006). These amplified tags are then subjected to high-throughput sequencing such as pyrosequencing using a 454 sequence analyzer (http://www.454.com/). Current 454 technology provides from 400-600 bp sequence reads per fragment, allowing complete coverage of each amplicon. Upwards of 100,000 individual sequences can be obtained from a single 454 experiment. The primers used to perform the initial tag amplification contain specific identifier sequencers or "bar codes" outside of the region of the primer that anneals to the target sequence. These bar codes allow correlation of the sequences, obtained *en masse*, back to an original sample. For example, bar coding allows

the simultaneous sequencing of greater than 100 individual ticks in a single experiment, generating hundreds of individual 16S rRNA tag sequences that can be correlated back to each specific source tick. Each sequence is then analyzed using the sequence databases (such as the RDP described above) to provide phylogenetic information about each microbe. Because of the depth of sequencing afforded, the number of sequences matching a specific taxon also provides information of the relative abundance of the microbe within the original sample. In our own studies we have found good agreement between abundances determined by tag sequencing and those determined by more direct assays such as targeted Q-PCR (Silvanose et al. in preparation).

All of these molecular approaches depend on PCR to amplify targeted genes and thus are all subject to any biases introduced by the PCR reaction itself. It is clear that there are no truly universal primer sets, and that in any given experiment it is possible to miss an important community member simply because of poor amplification. Conversely, some microbes may be overrepresented due to aberrantly efficient PCR. High-throughput sequencing may offer the answer here as well. New sequencing technologies, such as that provided by Solexa sequencing on Illumina instruments (http://www.illumina.com/technology/sequencing_technology.ilmn) can generate staggeringly high numbers of sequences, now up to 2 x 10^{11} bp in a single experiment. At a read length of roughly 100 bp this represents greater than 10^{9} individual sequences (generally much shorter in length than obtained by 454). With Illumina sequencing and other emerging technologies the ability to acquire sequence information from samples will no longer be the rate-limiting issue (Morozova and Marra 2008). With this sequencing power it may be possible to analyze total DNA from ticks directly, obtaining the tick genome and its microbial colonist's genomes, without the need for PCR, and avoiding the bias described above. As with many of these sequence-based technologies, the true challenge will be analyzing the bioinformatic data to obtain reliable information on the microbiome.

Molecular approaches have also begun to show great utility in studying tick host-microbiome interactions. Several groups have generated expressed sequence tag (EST) cDNA libraries from ticks that provide information not only on gene content, but also gene activity at the time of sampling (Wang et al. 2007; Hill and Gutierrez 2000). These libraries can also provide information about the microbiome. Our own analysis of several *A. americanum* EST libraries revealed a strikingly large percentage of cDNAs derived from a bacterial symbiont, indicating active expression of symbiont genes (Smith et al., in preparation). The sequence information gained from such libraries also facilitates construction of DNA microarrays to analyze gene expression (Colbourne et al. 2007). Whole genomic sequence projects for *Ixodes scapularis* and *Boophilus microplus* are well underway (Pagel Van Zee et al. 2007; Guerrero et al. 2006), and this information should provide even more comprehensive information for construction of DNA microarrays. With these microarrays, the expression patterns induced in ticks under a variety of conditions, including variable composition of the microbiome, may be monitored readily (Rodriguez-Valle et al. 2010). Even more powerful for these purposes may be massively parallel sequencing of transcripts, or RNASeq, using next generation sequencing technology to generate information on the genes being expressed and their level of expression (Ronning et al. 2010). This is as yet a new approach and we are not aware of its application to ticks or tick-borne disease, but the technique has great potential.

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Microbial Communities of Ticks

Over the past two decades, research has revealed unsuspected microbial diversity in arthropods. For example, it is estimated that *Wolbachia* occurs in over 65% of all insect species (Hilgenboecker et al. 2008) and other prokaryotes (e.g. *Cardinium*, *Arsenophonus*, *Rickettsia*) are also highly represented (Perlman, Hunter, and Zchori-Fein 2006; Duron, Wilkes, and Hurst 2010; Duron et al. 2008; Weinert et al. 2009; Novakova, Hypsa, and Moran 2009). These microbes are often associated with reproductive alterations in hosts such as feminization, induced parthenogenesis or reproductive incompatibilities (Werren, Baldo, and Clark 2008). More specific arthropod families or genera are often associated with other bacterial endosymbionts such as *Buchnera* in aphids that play a role in nutrition of their hosts by provisioning critical amino acids (Douglas 1998; Oliver et al. 2010). Other insect groups (e.g. beetles, cockroaches, termites) are also associated with specific groups of microbes with enzymatic capabilities for the digestion of cellulose-rich food materials (Dillon and Dillon 2004; Vasanthakumar et al. 2008; Sabree, Kambhampati, and Moran 2009). A growing literature suggests that bacterial symbionts can also play an important role in host defense against biotic enemies (Oliver, Moran, and Hunter 2005; Oliver et al. 2009; Jaenike et al. 2010), and also against abiotic stresses such as heat and cold (Montllor, Maxmen, and Purcell 2002; Neelakanta et al. 2010). Except for pathogens of humans and domestic animals, the functional role and impact of most microbial associations in ticks is unknown. It is likely that some endosymbionts play a nutritional role during blood feeding.

Most attention has been given to pathogenic bacteria vectored by ticks but they are also capable of transmitting pathogenic piroplasms (e.g. *Babesia* and *Theileria*) (Florin-Christensen and Schnittger 2009; Bishop et al. 2004) and a variety of viral pathogens. For example, tick-borne encephalitis is major human health threat worldwide (LaSala and Holbrook 2010; Charrel et al. 2004) and there is growing concern over deer tick or Powassan virus (Flavivirus) in *Ixodes*-endemic areas (Tokarz et al. 2010; Ebel 2010). In addition to tick-borne Flaviviruses, Colorado tick fever is caused by a Coltivirus transmitted by *Dermacentor andersoni* in the western United States and Canada (Brackney et al. 2010). Other pathogenic viruses could potentially be transmitted by ticks but standard methodologies for detecting bacteria would not detect them. The panviral Virochip approach would represent one possible method for quickly screening tick samples for viruses. Their blood-feeding habitat makes ticks potential vectors for a wide range of blood-borne pathogens.

Ticks may be co-infected by multiple pathogens (Schouls et al. 1999; Mixson et al. 2006; Moreno et al. 2006; Tokarz et al. 2010). Moreover, because of the high prevalence of vertically-transmitted endosymbionts in ticks, including multiple endosymbionts within the same tick (Scoles 2004; Goethert and Telford 2005; Carmichael and Fuerst 2006; Clay et al. 2008), pathogen infections almost always co-occur with resident endosymbionts (Yabsley et al. 2009; Jasinskas, Zhong, and Barbour 2007; Sun et al. 2000; Niebylski et al. 1997; Noda, Munderloh, and Kurtti 1997). Prior studies of the relationships among ticks, vertebrate hosts and pathogens have generally given little consideration to how microbial interactions and the entire microbial community within ticks, including endosymbionts and other microbes of unknown function, (Table 1). These associations might affect the colonization, transmission and virulence of human or animal pathogens

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TABLE 1 Tick-Borne Bacteria and Human Diseases.

Tick Genus	Species	Bacteria	Human Disease	Reference
Ixodes	Is, Ir	*Borrelia burgdorferi*	Lyme Disease	Burgdorfer et al. 1982
	Is	*Anaplasma phagocytophila*	Anaplasmosis	Belongia et al. 1997
	Is, Ir	*Rickettsia* symbiont	No known	Noda, Munderloh, and Kurtti 1997
	Is, Ip	*Arsenophonus* symbiont	No known	Grindle et al. (Unpublished)
	Ir	*Cytophaga* symbiont	No known	Morimoto, Kurtti, and Noda, 2006
	Ir	*Midichloria mitochondrii*	No known	Beninati et al. 2004
	Ir	*Diplorickettsia massiliensis*	No known	Mediannikov et al. 2010
Dermacentor[b]	Dv	*Rickettsia rickettsii*	Rocky Mountain Spotted Fever	Shepard and Goldwasser, 1960
	Dv, Da	*Rickettsia montana*	No known	Steiner et al., (Unpublished)
	Dv, Da	*Francisella* symbiont	No known	Sun et al. 2000
	Dv, Da	*Arsenophonus* symbiont	No known	Grindle et al. 2003
Amblyomma[c]	Aa	*Borrelia lonestari*	Southern Tick-Associated Rash Illness (STARI)	Varela et al. 2004
	Aa	*Ehrlichia chafeensis*	Ehrlichiosis	Anderson et al. 1991
	Aa	*Rickettsia amblyommii*	No known	Clay et al. 2008
	Aa	*Arsenophonus* symbiont	No known	Clay et al. 2008
	Aa	*Coxiella* symbiont	No known	Jasinskas, Zhong, and Barbour 2007, Klyachko et al. 2007

[a] Is, I. scapularis; Ir, I. ricinus; Ip, I. Pacificus
[b] Dv, D. variabilis; Da, D. andersoni
[c] Aa, A. americanum
[d] The mammalian pathogens Coxiella burnettii (Q-Fever) and Francisella tularensis (Tularemia) can be occasionally harbored and transmitted by multiple tick species

Ticks could become co-infected by pathogens while consuming a single blood meal containing multiple pathogens, or by transfer of pathogens between co-feeding ticks (Piesman and Happ 2001). It is less likely that ticks become infected by a diversity of pathogens from sequential feeding on multiple animals given that the hard tick life cycle includes only three blood meals well-separated in time. In contrast, co-infections of vertebrates by tick-borne pathogens could easily result from sequential and independent tick bites given that a large animal host could have a tick burden in the hundreds or thousands (Ginsberg 2008). Moreover, hosts may be bitten by ticks co-infected with multiple pathogens as described above. Human co-infections are most likely to arise from the bite of a single co-infected tick. Simultaneous infections by multiple tick-borne pathogens occur frequently in mammalian hosts, including humans. For example, of 96 patients in Wisconsin and Minnesota infected with *Borrelia burgdorferi*, five were co-infected with *Anaplasma phagocytophilum*, two with *Babesia microti* and two with all three pathogens (Mitchell, Reed, and Hofkes 1996). In New York, 60-90% of patients diagnosed with Human Granulocytic Anaplasmosis (*A. phagocytophilum*) tested positive for *B. burgdorferi*, a higher than expected rate based on pathogen prevalence (Wormser et al. 1997, see also Mitchell, Reed, and Hofkes 1996). Tick-borne co-infections may result in increased severity and duration of illness (Alekseev et al. 2001; Nyarko, Grab, and Dumler 2006)

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and misdiagnosis resulting from symptom overlap (Belongia et al. 1997). Co-infections with tick-borne pathogens have also been reported from domestic and wild animals including dogs (Kordick et al. 1999), deer (Little et al. 1998), rodents (Zeidner et al. 2000), cattle (Marufu et al. 2010) and horses (Parola, Davoust, and Raoult 2005).

Co-infections within ticks and competitive or facilitative interactions among microbes can affect the colonization and transmission of other tick-borne pathogens (Lively et al. 2005; Burgdorfer, Hayes, and Mavros 1981; Macaluso et al. 2002; de la Fuente, Blouin, and Kocan 2003; Ginsberg 2008) and the severity of ensuing disease (Korenberg 2004). For example, *Ixodes* ticks may be simultaneously infected by *B. burgdorferi* and other *Borrelia* species, *B. microti, A. phagocytophilum, Bartonella henselae* and Powassan virus (Tokarz et al. 2010; Goodman, Dennis, and Sionenshine 2005). Similarly, *Amblyomma* ticks may simultaneously harbor *Borrelia lonestari, Ehrlichia spp.*, and *Rickettsia amblyommii* (Heise, Elshahed, and Little 2010; Clay et al. 2008; Castellaw et al. 2010). If colonization of ticks by a particular microbe leads to the exclusion or facilitation of other microbes, this would be manifested as a significant statistical deviation from random co-occurence. For example, Mather et al. (Mather, Riberiro, and Spielman 1987) suggested that the agents of Lyme Disease and Babesiosis occurred together in ticks more frequently than expected. In contrast, Schauber et al. (Schauber et al. 1998) found that infection of *I. scapularis* by *B. burgdorferi* and *A. phagocytophilum* were independent of each other. Likewise, *A. phagocytophilum* and *B. burgdorferi* were acquired by mice regardless of their prior infection status by the opposite agent and were transmitted independently (Levin and Fish 2000).

Analysis of microbial exclusion or facilitation requires explicit reporting of co-infection rates. In a recent meta-analysis, 44% of the *Ixodes* tick populations (8 of 18) meeting criteria for inclusion significantly deviated from expected co-infection frequencies under the assumption of independent infection of *A. phagocytophilum* and *B. burgdorferi* (Civitello, Rynkiewicz, and Clay 2010; Ginsberg 2008). In contrast, there was no evidence of deviation from expected rates of co-occurrence of five microbial taxa in *Amblyomma americanum* (Clay et al. 2008). However, the *Coxiella* endosymbiont occurred at 100% prevalence and two recognized human pathogens (*E. chaffeensis* and *B. lonestari*) occurred at very low frequencies, leading to limited statistical power to detect deviations from independent association.

Competition and crossover of vertebrate host immune response may be greatest between closely related strains (Barthold 1999; Pal et al. 2001). For example, infection by some Spotted Fever Group *Rickettsia* in *Dermacentor variabilis* prevents establishment and vertical transmission of related *Rickettsia* (Macaluso et al. 2002) see also (Burgdorfer, Hayes, and Mavros 1981). Price (Price 1953) described a different form of interaction between virulent and non-virulent rickettsiae where guinea pigs injected with both forms were protected from the effects of the virulent rickettsiae, possibly as a result of immunological cross-protection. We expect that vertically-transmitted tick endosymbionts should inhibit or exclude pathogens if those pathogens cause some harm to tick hosts (e.g. Niebylski, Peacock, and Schwan 1999). It is in the evolutionary interest of vertically-transmitted endosymbionts to exclude pathogens from the tick microbial community because infection by a virulent pathogen condemns that endosymbiont community to extinction (Lively et al. 2005). It is clear that complex communities of microorganisms can coordinate activities within hosts or interfere with other microbes via cell-cell communication, and such mechanisms may be relevant to the interactions between tick-borne endosymbionts and pathogens (Fuqua and Greenberg 2002).

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Overall, these studies demonstrate that ticks harbor a diversity of pathogens and symbionts, potentially allowing for ecological interactions among microbes within ticks. Microbial interactions could affect pathogen prevalence and transmission within tick populations. The role of microbial interactions in the organization of microbial communities within vectors and hosts needs further critical evaluation. An important first step is the evaluation and enumeration of microbial diversity within ticks.

Diverse Microbiome of Ticks

In preliminary studies of eastern North American ticks, we have examined the prokaryotic diversity of ticks by 16S rRNA tag sequencing using a 454 approach for amplicons from DNA extracts of *A. americanum*, *D. variabilis* and *I. scapularis* collected from the wild. All individuals were adult, questing ticks that were rigorously surface sterilized before DNA extraction and sample preparation. The proportion of annotated sequences corresponding to the 10 most frequent taxa are presented in Fig. 3 for each species. The number of sequences from a given taxon is presumed to reflect the density of that microbe within the tick. For *A. americanum*, the most abundant sequences were from the *Coxiella* endosymbiont (approx. 40% of all sequences) with *Rickettsia* being the second most common (approx. 5% of total), in good agreement with our direct probing data (Clay et al. 2008). We could not distinguish species of *Rickettsia* (and other groups) with accuracy so only present generic classifications. More than 40% of the identified sequences were from a large variety of other microbes. For *D. variabilis*, an *Arsenophonus* endosymbiont was the most frequently identified prokaryote followed by *Methylobacterium* and *Francisella*. Nearly 40% of the identified sequences were from a large number of rare taxa. Finally, for *I. scapularis*, *Rickettsia* represented nearly 75% of the total sequences with *Bacillus* making up approximately 10% of the total. The “other” category was relatively small in *Ixodes*.

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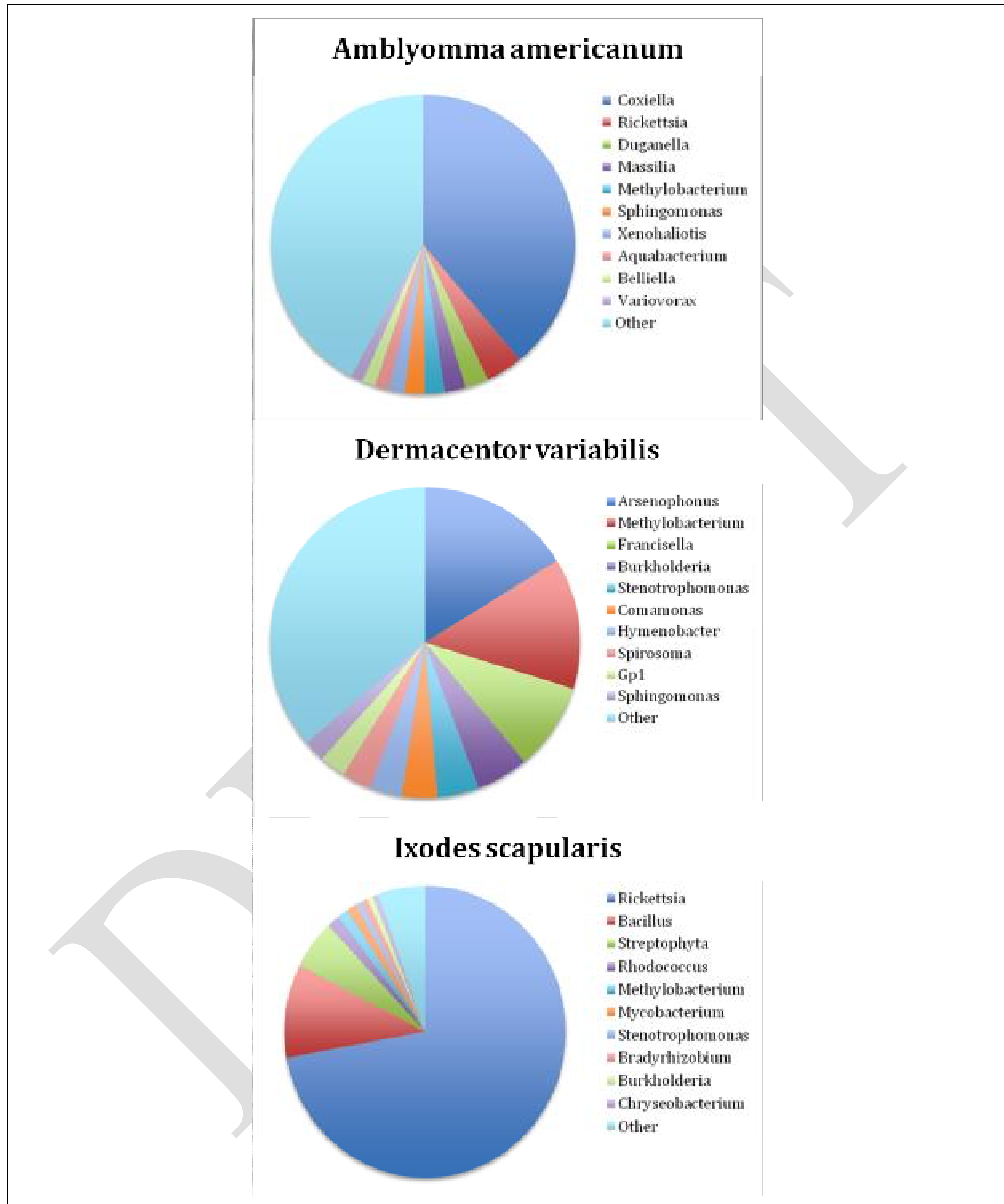

FIGURE 3 Density of annotated bacterial sequences in *Amblyomma americanum* (N=32), *Dermacentor variabilis* (N=22) and *Ixodes scapularis* (N=19) based on 454 sequencing. All ticks were collected from various sites in Indiana. The top 10 most abundant sequences are given for each species; other indicates all remaining sequences.

The prevalence of prokaryotes across individual ticks provides another measure of the tick microbiome (Fig. 4). 100% of the sampled*A. americanum* ticks were host to *Coxiella* with

over 90% host also to *Methylobacterium* and *Sphingomonas*. 75% were infected by *Rickettsia*. Notably, *Rhizobium*, usually associated with nitrogen fixation, was also detected in 75% of the sampled ticks. For *D. variabilis*, the three most prevalent microbes were *Methylobacterium*, *Francisella* and *Sphingomonas*, each found from 19 of 22 (86%) sampled ticks. Three-quarters of *I. scapularis* hosted *Rickettsia* with no other microbe found in greater than 53% of the samples. *Bradyrhizobium*, another known N-fixing group, was found in 37% of the sampled *Ixodes* ticks. Although these findings are preliminary and subject to modification based on additional experiments, they do clearly indicate the potentially significant microbial diversity in ticks.

Known human pathogens were occasionally detected (data not shown because of their low density and prevalence) including *Ehrlichia* from *Amblyomma* and *Borrelia* from *Ixodes*. In *Amblyomma* we also occasionally detected sequences from *Cardinium*, another commonly-reported arthropod endosymbiont (Duron et al. 2008) that has never before been reported from ticks. *Arsenophonus* is another widespread insect endosymbiont (Novakova, Hypsa, and Moran 2009) that has recently been reported from several tick species (Dergousoff and Chilton 2010; Grindle et al. 2003; Clay et al. 2008). While our results are preliminary and need to be repeated with a larger sample of tick species and individuals, they clearly point to the fact that the dominant members of the tick microbiome are endosymbionts and/or microbes of unknown specificity and function. It is likely that some of these microbes play a nutritional role by helping to provision critical amino acids, vitamins, or otherwise help ticks survive on a limited diet of blood. Parallel microbiome studies of sap-sucking insects point to a critical role for nutritional endosymbionts, and a common incidence of convergent evolution of these symbionts (Sabree, Kambhampati, and Moran 2009; Oliver et al. 2010).

Future Directions

Ticks represent a compelling yet challenging system for the study of microbiomes and microbial interactions. They require blood meals prior to molting, their symbionts are difficult to cure and to deliberately inoculate, and their genomes are highly complex. The microbes that colonize ticks also can be very difficult to work with since many have not yet been cultivated or are obligate intracellular symbionts. Little is currently known about the roles and activities of many of these microorganisms. Additionally, they can have fluid genetic content, and the features which separate a benign commensal from a significant pathogen are not always clear or well understood. Now that it is clear that there can be multiple microorganisms colonizing the same tick and even the same tissues, the prospects for genetic exchange between these microbes is quite distinct, and it is conceivable that an otherwise benign commensal microbe might acquire virulence functions through this route. Likewise, synergistic or antagonistic interactions between microbes may be manifested by the emergence of new polymicrobial diseases or, alternatively, the decline of a current disease agent.

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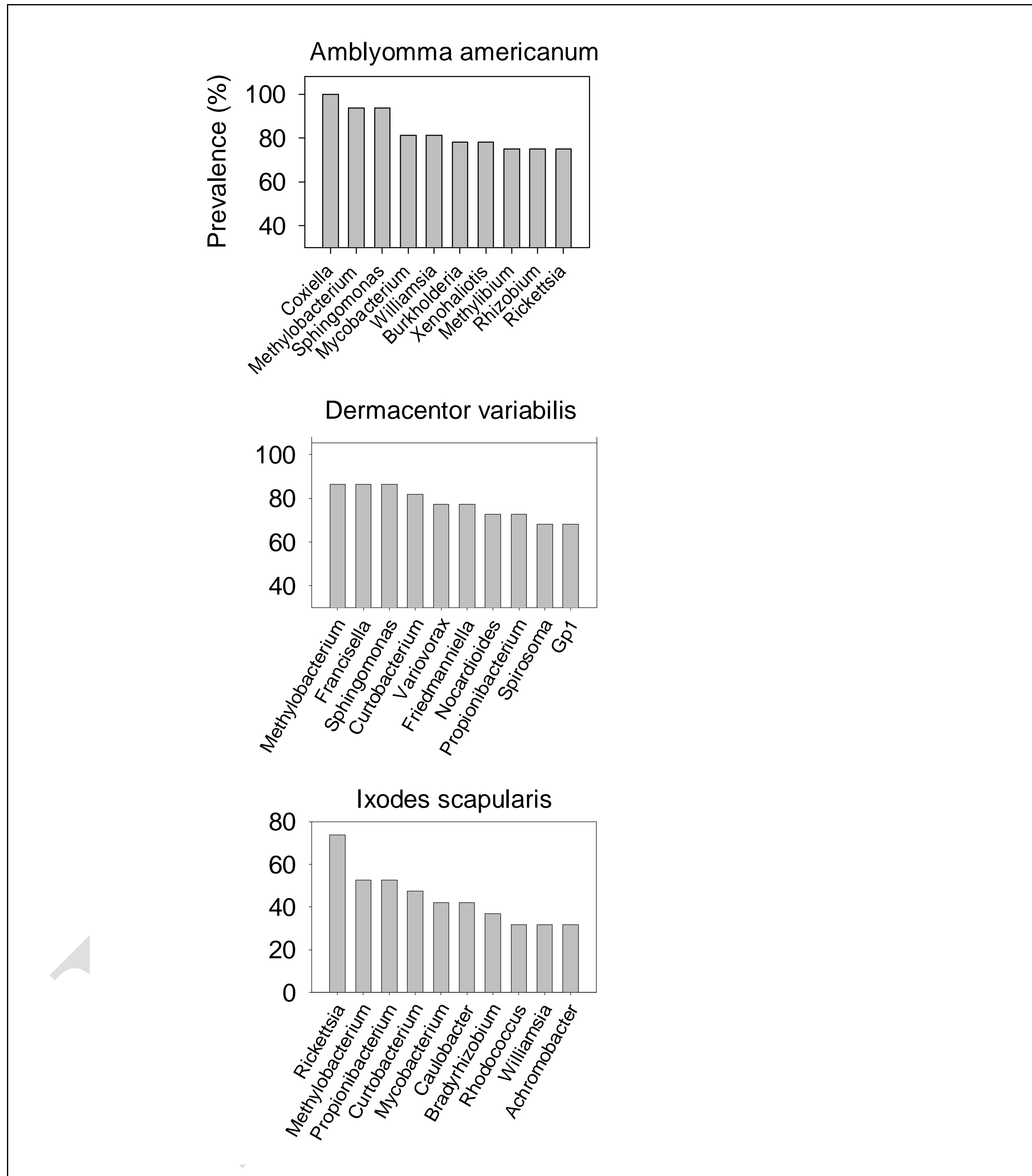

FIGURE 4 Density of annotated bacterial sequences in *Amblyomma americanum* (N=32), *Dermacentor variabilis* (N=22) and *Ixodes scapularis* (N=19) based on 454 sequencing. All ticks were collected from various sites in Indiana. The top 10 most abundant sequences are given for each species; other indicates all remaining sequences.

Although more efficient arthropod systems exist for fundamental investigations into microbial-host interactions, few have the human health impact of ticks and the microbes they vector. Ultimately, it is the importance of ticks to human health that drives active research in this area rather their utility as a model system. The increasing availability of genomic information for

ticks and tick-associated microbes creates significant opportunities to broaden the range of analyses that can be performed. Several tick-vectored pathogens have had their genomes sequenced (Seshadri et al. 2003) and several whole tick genome sequences should be forthcoming (Guerrero et al. 2006; Pagel Van Zee et al. 2007). Genomic sequencing of non-pathogenic symbionts is thus far less common, but would add to the understanding of the tick-borne community, affording opportunities for comparative genomics between related pathogens and non-pathogens, and yielding insights into acquisition and transmission processes. In addition, the ability to simultaneously evaluate tick gene expression as well as those of their resident microbiota, through DNA microarrays and RNASeq, should begin to unravel these tight and stable arthropod-microbe interactions.

As with other efforts rooted in genomic science a major hurdle lies within the bioinformatics. Acquisition of sequence or expression data is no longer rate limiting, but rather the ability to distill the potentially massive amount of data down to manageable segments or significant patterns is very challenging, and efficient progress will require interdisciplinary teams of microbiologists, epidemiologists and bioinformaticians.

While technological advances and deep sequencing has revealed unsuspected microbial diversity, many basic questions remain unanswered. What are the evolutionary origins and means of spread of these microbes? What is their functional role or are they simply highly abundant guest commensals? For example, *Methylobacterium* was highly represented in all tick sequences and occurred at very high prevalence in all tick species examined (Fig. 3 and 4). What is the functional role of the highly abundant endosymbionts such as *Arsenophonus*, *Coxiella*, *Francisella* and *Rickettsia*? What distinguishes hereditary endosymbionts from virulent human pathogens (e.g. *Coxiella* endosymbiont of *A. americanum* vs. *C. burnetti* (Jasinskas, Zhong, and Barbour 2007; Klyachko et al. 2007; Seshadri et al. 2003)? Could horizontal gene exchange between related pathogens and endosymbionts give rise to new virulent pathogens? More generally, are there characteristic microbial communities associated with different tick species and what regulates the structure of these tick-associated microbial communities? Addressing these questions will require more genomic data from non-pathogens combined with efficient inoculation and disinfection systems. Greater understanding of the dynamics and organization of tick-associated microbial communities may also contribute to the development of more accurate epidemiological and disease risk models.

The increasing homogenization of Earth's biota and human domination of terrestrial ecosystems may be increasing, rather than decreasing, human health risks. Ticks and their pathogens are highly dispersible and thrive in many human-dominated habitats. Increasing wildlife populations, such as deer and turkey, may also contribute increasing risk of tick encounters (Childs and Paddock 2003). For example, annual incidence of Lyme disease is increasing despite greater awareness and prompt medical responses. Climate change may further alter geographical ranges of ticks, pathogens and vertebrate hosts (Randolph 2010), potentially leading to host and vector shifts of tick-associated microbes and the composition of their microbial communities. Tick-borne pathogens are just one component of larger, diverse microbial communities. Genetic exchange between pathogens and symbionts, exchange of virulence factors, new mechanisms for contagious transmission and new host associations all should be considered in light of larger scale ecological and environmental changes.

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