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Discovery and Surveillance of Tick-Borne Pathogens

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Abstract

Within the past 30 yr molecular assays have largely supplanted classical methods for detection of tick-borne agents. Enhancements provided by molecular assays, including speed, throughput, sensitivity, and specificity, have resulted in a rapid increase in the number of newly characterized tick-borne agents. The use of unbiased high throughput sequencing has enabled the prompt identification of new pathogens and the examination of tick microbiomes. These efforts have led to the identification of hundreds of new tick-borne agents in the last decade alone. However, little is currently known about the majority of these agents beyond their phylogenetic classification. Our article outlines the primary methods involved in tick-borne agent discovery and the current status of our understanding of tick-borne agent diversity.

Key words: next-generation sequencing, microbiome, pathogen discovery

Ticks are vectors of the greatest diversity of vertebrate pathogens worldwide, in large part due to their hematophagous ectoparasitic life-style and promiscuity in host selection (Jongejan and Uilenberg 2004, McCoy et al. 2013, Estrada-Peña and De La Fuente 2014). Since the discovery of the first tick-borne agent in the late 19th century, over 20 bacterial, viral, and protozoan human tick-borne pathogens have been identified in the United States alone (Eisen et al. 2017, Smith and Kilborne 1893, Center for Disease Control and Prevention Diseases 2019). The discovery of most of these agents was correlated with the rapid tick expansion for several important human-biting tick species that occurred during the last century as well as advances in detection methods that were aimed at identification of new agents (Telford et al. 1997, McMullan et al. 2012, Kosoy et al. 2015, Monzón et al. 2016, Sonenshine 2018, Eisen and Paddock 2020). Within the past 30 yr, the implementation of molecular assays for tick-borne pathogen surveillance and discovery have had a major impact on our understanding of the diversity of tick-borne agents and their contribution to human disease. In particular, the employment of next-generation sequencing (NGS) has identified a wide range of novel tick-borne pathogens and facilitated studies of the tick microbiome that resulted in the discovery and characterization of hundreds of new tick-associated microbes (Tokarz et al. 2014b, Li et al. 2015a, Shi et al. 2016a, Bouquet et al. 2017, Pettersson et al. 2017, Harvey et al. 2018, Tokarz et al. 2018b, Sameroff et al. 2019). In addition to enhancing our knowledge of tick-borne microbial diversity, NGS studies have provided invaluable insight into evolutionary relationships among tick-associated agents, identified new targets for diagnostic assays, and provided new approaches to understanding tick-borne diseases (TBDs) (McMullan et al. 2012,

Li et al. 2015a, Tokarz et al. 2019). Our article will illustrate the remarkable improvements in the identification of tick-borne agents achieved with molecular assays and outline the current state of our understanding of tick-borne agent diversity.

Early Strategies for Tick-Borne Agent Discovery

Prior to the advent of molecular biology, tick-borne agents were historically identified through microscopy, isolation in culture or serology (Cowdry 1925, Burgdorfer et al. 1982, Benach et al. 1983, La Scola and Raoult 1997, Raoult and Roux 1997, Mans et al. 2015). Among the initial tick-borne agents identified were the apicomplexan parasites *Babesia* and *Theileria* that were visualized in blood smears from infected cattle (Mans et al. 2015, Smith and Kilborne 1893). Studies of Howard Ricketts were integral in recognizing tick transmission of the causative agent of Rocky Mountain Spotted Fever (RMSF), subsequently named *Rickettsia rickettsii* (Ricketts 1906, 1991; Solheim 1949; Parola et al. 2005). Serologic methods such as complement fixation and immunofluorescence assays (IFA) supplemented microscopy studies while transmission of the presumed etiologic agents between vector ticks and naïve vertebrate hosts were used to fulfill proof of causation (Solheim 1949, Raoult and Roux 1997, Mans et al. 2015). By mid 20th century, advances in tissue culture methods facilitated the isolation of many of the most common tick-borne pathogens and enabled the study of tick-borne viruses. Multiple combined approaches were often used for identification of novel agents. Large scale global programs aimed at the discovery of new viruses led to the isolation of a wide range of

tick-borne viruses, some of which were subsequently characterized through serology and electron microscopy (Downs 1982). *Borrelia burgdorferi* sensu stricto (s.s.) was identified as the tick-borne agent of Lyme borreliosis by visualizing spirochetes in blood from patients and vector ticks and demonstrating serologic reactivity to cultured spirochetes with sera from Lyme borreliosis patients (Burgdorfer et al. 1982, Benach et al. 1983).

Although these approaches were successful in the identification of the agents responsible for most well-known TBDs, the discovery of new tick-borne agents was often compromised by limitations inherent to non-molecular methods. For some agents, isolation in culture proved challenging (Anda et al. 1996, Hue et al. 2013, Tokarz et al. 2014b). This limitation particularly applied to the constituents of the tick virome, where very little was learned about viral diversity for even the most clinically relevant tick species. Until recently, only a single virus was isolated from *Ixodes scapularis* Say, whereas we now know this tick has a very diverse virome (Telford et al. 1997, Tokarz et al. 2014b, Tokarz et al. 2018b). In addition, tick-borne viruses that were isolated in viral surveillance studies were often not characterized further (Palacios et al. 2013, Walker et al. 2015). Cross-reactivity of serologic assays was often a problem for accurate agent identification. This was especially evident in work with *Rickettsia*, where nonpathogenic bacterial endosymbionts could be mistaken for closely related pathogenic species, and serologic cross-reactivity among spotted fever *Rickettsia* likely impeded the identification of new distinct species (Parola et al. 2005).

Although these classical methods are still in use today, by the early to mid-1990s, polymerase chain reaction (PCR) mostly supplanted them as the primary tool for detection of tick-borne agents. The advantages of PCR, including low cost, speed and high throughput, made it a more effective tool for tick-borne pathogen surveillance. Dideoxy sequencing of PCR products provided genomic sequence data that could be used to more clearly differentiate microbial species and improve taxonomic classification. Over time, the utility of PCR was further enhanced by additional improvements in assay design, experimental methods. Although initial tick-borne pathogen surveillance studies often utilized tick pools for PCR analysis, advances in nucleic acid extraction facilitated the study of individual ticks (Johnson et al. 1992, Steiner et al. 1999). Laborious manual nucleic acid extractions have been increasingly supplanted by automated extraction platforms that can rapidly provide nucleic acid template material from ≥ 24 individual tick samples (Exner and Lewinski 2003, Tokarz et al. 2010, Tokarz et al. 2018b, Sameroff et al. 2019). The implementation of multiplex PCR assays that utilize primer pairs targeting >1 agent further reduced the cost and speed of testing (Tokarz et al. 2010, Hojgaard et al. 2014, Tokarz et al. 2017, Reller and Dumler 2018, Buchan et al. 2019, Sanchez-Vicente et al. 2019). As a result of these modifications, tick-borne pathogen surveillance studies now often report the testing of hundreds to thousands of tick samples (Tokarz et al. 2010, Aliota et al. 2014, Prusinski et al. 2014, Edwards et al. 2015, Hutchinson et al. 2015, Johnson et al. 2018, Sanchez-Vicente et al. 2019). Numerous surveillance studies have been reported from different countries and continents (Chalada et al. 2018, Cull et al. 2018, Guo et al. 2019). In the United States, the majority of surveillance work has focused on *I. scapularis* due to its association with *Borrelia burgdorferi* s.s. as well as other human pathogens. These studies established *B. burgdorferi* as the most prevalent tick-borne pathogen, with infection rates of 15 to 25% in nymphs and 40–70% in adult ticks (Tokarz et al. 2010, Aliota et al. 2014, Prusinski et al. 2014, Tokarz et al. 2017). Other agents transmitted by *I. scapularis*, such as *Anaplasma phagocytophilum* and *Babesia microti*, are usually detected in up to 20% of nymphs, while $<5\%$ are infected with *B. miyamotoi* and Powassan

virus. Tick surveillance studies also revealed that approximately up to 5% of nymphs and up to 15% of adult ticks simultaneously carry multiple pathogens (Tokarz et al. 2010, Aliota et al. 2014, Prusinski et al. 2014, Tokarz et al. 2017, Sanchez-Vicente et al. 2019). These findings correlate with serologic data from patients with TBDs, where probable co-infections have been documented going back several decades (Filstein et al. 1980, Benach and Habicht 1981, Burgdorfer et al. 1982, Krause et al. 1996, Hilton et al. 1999, Horowitz et al. 2013, Curcio et al. 2016, Tokarz et al. 2018a).

PCR as a Tool for Tick-Borne Agent Discovery

Although PCR is typically employed to target and amplify a single nucleic acid sequence, modifications in primer design have enabled the use of PCR as a discovery tool. Instead of targeting a single sequence, alignments of multiple sequences from different agents are used to identify conserved genomic loci within a larger taxonomic group such as a genus or family (Tokarz et al. 2010, Toledo et al. 2010, Tadin et al. 2016, Sanchez-Vicente et al. 2019). PCR primers are then designed within the most conserved regions with the assumption that they will recognize multiple (or all) members within the targeted taxonomic unit. Where necessary, degeneracies can be incorporated into these primer sequences to include divergent sequences. Once amplified, the PCR product is typically sequenced by standard dideoxy sequencing and the agent is classified through homology searches in GenBank (Fukunaga et al. 1995, Pancholi et al. 1995, Tokarz et al. 2010, Sanchez-Vicente et al. 2019). New agents can also be uncovered by examination of melting temperature of PCR products that would indicate the presence of novel sequences, an approach that led to the discovery of novel pathogenic species of *Ehrlichia* and *Borrelia* in patients with TBDs (Bell and Patel 2005; Pritt et al. 2011, 2016).

The introduction of PCR has had a profound impact on the identification of tick-borne agents. Many of the previously isolated but uncharacterized tick-borne viruses could now be identified, while newly emerging pathogenic viruses were rapidly characterized (Telford et al. 1997, Honig et al. 2004). Phylogenetic analyses of genomic sequences obtained with PCR led to the reclassification of many known tick-borne agents. One prominent example is the bacterial agent of human granulocytic anaplasmosis that initially was identified by microscopy and assigned as a species of *Ehrlichia*. Through molecular analysis of multiple genetic loci, this agent was shown to be distinct from *Ehrlichia* and was eventually placed within another genus with a new name, *Anaplasma phagocytophilum* (Chen et al. 1994, Dumler et al. 2001). PCR was frequently employed to examine the complex etiology of rickettsiosis and to differentiate highly similar species that could not be resolved through serology (Shapiro et al. 2010). It also was used to determine the presence of *Rickettsia* in ticks, especially *R. rickettsii*, a species historically presumed to be the primary cause of spotted fever disease in the United States. These studies occasionally failed to detect *R. rickettsii* in areas endemic for spotted fever but detected other species of *Rickettsia* that have been linked with human disease, including *R. amblyommatis* and *R. montanensis* (Apperson et al. 2008, Mcquiston et al. 2012, Wood et al. 2016, Sanchez-Vicente et al. 2019). These results led to the hypothesis that these other species of *Rickettsia* may be responsible for a portion of spotted fever cases typically attributed to *R. rickettsii* (Stromdahl et al. 2011, Gaines et al. 2014). In addition, PCR detection of pathogens in field-collected ticks has been used to identify tick species potentially serving as vectors of TBD (Pancholi et al. 1995; Savage et al. 2013, 2017).

A limitation of PCR assays is that only closely related agents can typically be identified with this approach. Historically, PCR assays aimed at agent discovery were focused on a few microbial genera or families that included known vertebrate pathogens and this precluded the discovery of agents with divergent genomes. In addition, by targeting highly conserved genetic loci, PCR can provide limited genomic information and subsequent analyses could be insufficient to determine actual taxonomic relationships (La Scola et al. 2003).

Next-Generation Sequencing

Unlike standard dideoxy sequencing, which generates a single DNA sequence, NGS generates thousands to hundreds of millions of sequences, or reads, that are present in a sample (Liu et al. 2012, Goodwin et al. 2016). As a result, NGS sequencing generates large datasets that require a bioinformatic 'pipeline' for quality control of sequencing reads, subtraction of host reads, contig assembly, and homology searches that assign taxonomic identification to reads and contigs (Chevreux et al. 1999, Andrews and Bittencourt a 2010, Schmieder and Edwards 2011, Langmead and Salzberg 2012, Scholz et al. 2012, Li et al. 2015b). Often, microbial reads represent only a fraction of the total reads, and in the case of novel viral agents, these can have minimal homology to known sequences (Tokarz et al. 2014b, Li et al. 2015a, Tokarz et al. 2018b).

Two methods are used for metagenomic examination by NGS: amplicon sequencing and shotgun sequencing (Goodwin et al. 2016, Greay et al. 2018). Amplicon sequencing is a highly targeted approach that uses a consensus primer pair to amplify a specific, highly conserved region of a single gene that is further characterized through NGS. Because of its high sequence conservation in bacteria, the 16S rRNA has been the optimal target gene used for amplicon sequencing. The 16S rRNA gene consists of multiple overlapping constant and hypervariable regions. Primers designed to bind to constant regions are used to amplify all 16S rRNA sequences present in a sample. The diversity in the sample is determined by aligning the 16S rRNA PCR product to datasets comprising known rRNA sequences (Wu et al. 2008, Schloss et al. 2009, Caporaso et al. 2010, Quast et al. 2013, Cole et al. 2014).

The first metagenomic NGS study of tick-borne agents was published in 2011 (Andreotti et al. 2011). Numerous studies have been performed thereafter on a wide range of tick species worldwide (Carpi et al. 2011, Ponnusamy et al. 2014, Gofton et al. 2015, Rynkiewicz et al. 2015, van Treuren et al. 2015, Trout Fryxell and DeBruyn 2016, Gurfield et al. 2017). Although the 16S rRNA gene is an effective tool for the assessment of bacterial diversity, it does not detect viral or eukaryotic agents. In addition, 16S rRNA primers can be biased for one taxonomic group over another, whereas in other cases, because of the high sequence conservation, phylogenetic resolution may not be sufficient for discrete taxonomic assignments (Kim et al. 2011, Bonk et al. 2018).

An alternative approach, shotgun sequencing, uses a randomized set of short oligonucleotides for priming and amplification of all genomic material present in a sample (Bouquet et al. 2017, Greay et al. 2018, Tokarz et al. 2019). The primary advantage of this approach is that it enables the detection and identification of all bacterial, eukaryotic, and viral agents present and facilitates the assembly of complete genomes. This results in a more comprehensive characterization of all the agents present and improved taxonomic resolution. A disadvantage is that it tends to be more laborious and expensive, primarily due to the additional sample preparation required to enrich for microbial template through reduction of host nucleic acids

(Lim et al. 2014, Tokarz et al. 2014b, Carpi et al. 2015, Sameroff et al. 2019). Shotgun sequencing is also less sensitive than 16S rRNA PCR. In addition, the datasets generated by shotgun sequencing are much larger and more complex leading to increased computational needs for the bioinformatic analyses.

Molecular Assay Confounds

Accurate NGS assessment of the tick microbiome can be heavily dependent on experimental factors (Narasimhan and Fikrig 2015). The surface of ticks can harbor a broad range of transiently acquired microbes that are likely not part of their internal microbiome. Unless removed prior to nucleic acid extraction, sequences of these agents will be amplified, and can erroneously be included as a portion of the microbiome (Binetruy et al. 2019). Reports of metagenomic analyses of ticks often include bacteria that likely represent environmental contaminants. Protocols for removal of environmental contaminants may include multiple washes of ticks with alcohol, hydrogen peroxide, or bleach. Of these, bleach treatment has been reported to have the best results (Hoffmann et al. 2020). Other confounds may include the presence of sequences that originate from reagent or laboratory contaminants (Salter et al. 2014). Occasionally, entire viral genomes have been recovered by NGS from clinical specimens, only to determine that they originated from laboratory reagents (Xu et al. 2013, Asplund et al. 2019). Bacterial sequences, and in particular ribosomal sequences, often can be found in water, enzymes, and buffers used for amplification (Salter et al. 2014, Gruber 2015). Ultraviolet irradiation of plasticware and enzymatic digestion of buffers may be helpful in minimizing the presence of nucleic acid contaminants (Woyke et al. 2011).

Sub-optimal PCR assay design also may contribute to misleading results. Ribosomal genes have often been used as targets for PCR because of the availability of genetic sequences for primer design, high degree of conservation, and the presence of multiple copies in the bacterial genome. However, designing agent-specific assays can be challenging, particularly within ribosomal genes. All sequences of genetic near-neighbors must be considered in primer design to minimize the potential for amplification of closely related bacteria (Tokarz et al. 2019). One solution is to design primers within other less conserved non-ribosomal genes. Another approach is to perform a secondary assay targeting an alternative genetic locus followed by dideoxy sequencing of PCR products for confirmation (Tadin et al. 2016).

Tick-Borne Bacterial Pathogens

Bacterial species within the genera *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, and *Rickettsia* are responsible for the majority of reported human TBD worldwide (Parola and Raoult 2001, Eisen et al. 2017). *Anaplasma phagocytophilum*, the primary species of *Anaplasma* linked with human disease, is rapidly increasing in incidence in the United States (Dumler et al. 2001, Rosenberg et al. 2018). *Borrelia* species cause two distinct human diseases. Whereas species transmitted by ixodid (hard) ticks, cause Lyme borreliosis, argasid (soft) ticks transmit agents of relapsing fever (Steere et al. 2016, Talagrand-Reboul et al. 2018). The most common tick-borne disease in the Northern hemisphere is Lyme borreliosis. In North America, it is caused by *B. burgdorferi* and *B. mayonii*. *Borrelia afzelli*, *B. garinii*, and *B. burgdorferi* are the primary correlate agents of Lyme borreliosis in Eurasia. Some species of relapsing-fever *Borrelia* are also found in ixodid ticks,

including the pathogenic *B. miyamotoi* (Fukunaga et al. 1995, Armstrong et al. 1996). Despite a wide array of *Coxiella*-like bacteria identified in ticks, *Coxiella burnetii*, the agent of Q fever, is the only known tick-borne pathogen from this genus (Zhong 2012). Ehrlichiosis is a disease of human and domestic animals caused by infection with species of *Ehrlichia*, with *E. chaffeensis* the primary agent of human disease in the United States (Ismail and McBride 2017). *Francisella tularensis* is the cause of tularemia, a rare but potentially fatal disease (Telford and Goethert 2020). A wide range of *Rickettsia* species have been identified in ticks worldwide. Although the majority are presumed to be nonpathogenic, several species can cause spotted fever, a potentially severe and lethal disease (Parola et al. 2005, Eisen and Paddock 2020).

The diagnostic methods employed for detection of these agents in clinical samples vary. Although molecular assays may be useful for some, during the early acute stages, serologic assays, typically IFA or enzyme-linked immunosorbent assays, are most frequently used for diagnosis (Wormser et al. 2006, Biggs et al. 2016, Connally et al. 2016). For Lyme borreliosis, the exceedingly low bacterial burden in blood makes molecular assays impractical, and serology is used almost exclusively for laboratory diagnosis (Hinckley et al. 2014, Connally et al. 2016).

In addition to pathogenic bacteria, PCR and NGS studies revealed that ticks often harbor nonpathogenic endosymbiotic bacteria. These mainly belong to four genera, including *Coxiella*, *Francisella*, *Rickettsia*, and *Midichloria mitochondrii* (Ahanrarig et al. 2013). All have an obligate intracellular lifecycle and are highly abundant in ticks. In NGS tick microbiome studies, sequences attributed to endosymbionts often dominate those from other bacteria. The endosymbionts are passed transovarially and transtadially, and are usually highly prevalent in their hosts (Socolovschi et al. 2009). The *Coxiella* endosymbiont of *Amblyomma americanum* (L.) has been detected in 100% of examined ticks (Jasinskas et al. 2007). For other endosymbionts, the prevalence can vary depending on the geographic region and sex of the ticks examined (Cross et al. 2018, Tokarz et al. 2019). Although the roles the endosymbionts play in the tick life cycle are still unclear, their presence may be beneficial to the tick hosts. Endosymbionts have been shown to be essential for survival and fitness of *Dermacentor andersoni* Stiles and *A. americanum* (Zhong et al. 2007, Clayton et al. 2015). Some rickettsial endosymbionts synthesize essential nutrients that can supplement the tick host and also inhibit colonization with pathogens (Hunter et al. 2015; Bodnar et al. 2018, 2020).

16S rRNA studies of ticks have reported a variety of bacterial species at a substantially lower abundance compared to endosymbionts and pathogens. Although these may represent commensal bacteria, it is unclear if the detection of these agents represents stable or transient components of the microbiome or if these sequences originate from environmental or reagent contaminants (Salter et al. 2014, Tokarz et al. 2019). Most of these bacteria are extracellular and it's been suggested that intracellular bacteria may be the only real stable component of the microbiome for some ticks (Ross et al. 2018). Errors introduced during sequencing and insufficient bioinformatic analyses can overestimate the numbers of species identified by NGS (Tijssse-Klasen et al. 2010). In addition, some agents that are detected in ticks by molecular methods may not be a part of the actual microbiome, but rather represent the remains of a past bloodmeal (Allan et al. 2010, Landesman et al. 2019). Therefore, the presence of sequences from atypical agents at a low abundance needs to be verified by additional assays.

Tick Virome

Historically, the identification and characterization of tick-borne viruses has lagged considerably behind the discovery of cellular agents. The lack of a highly conserved consensus gene, such as 16S rRNA in bacteria, limited the utility of consensus PCR assays for discovery of tick-borne viruses (Lwande et al. 2013, Matsuno et al. 2015). Reverse transcriptase (RT)-PCR assays aimed at viral discovery were typically designed to target the viral RNA-dependent RNA polymerase (RdRp) (Maher-Sturgess et al. 2008, Zlateva et al. 2011). Despite the ubiquitous presence of genes encoding RdRp in RNA viruses, the poor sequence conservation, even among viruses within the same genus, limited the utility of this approach for discovery. In addition, these assays were based on alignments of the relatively few available sequences of members of a given genera or family, and overall this limited success in uncovering novel viral agents. The employment of tissue culture for virus isolation, although more costly and laborious, was more successful (McLean et al. 1962, Taylor et al. 1966, Downs 1982, St. George et al. 1984, Topolovec et al. 2003, Pinto Da Silva et al. 2005). An added benefit of this approach was the isolation of viruses that were capable of replication outside the vector host, suggesting the potential for transmissibility and vertebrate infection, especially when isolated in vertebrate cell lines such as Vero cells. However, virus isolation can frequently be unsuccessful, and viruses closely associated with their arthropod hosts were refractory to isolation in vertebrate cell lines. In retrospect, only a fraction of the components of tick viromes were isolated in tissue culture. In addition, after isolation, tick-borne viruses were often insufficiently characterized or misclassified due to the lack of adequate tools for molecular characterization (Taylor et al. 1966, Takahashi et al. 1982).

Shotgun sequencing enabled the identification of a wide range of tick-borne viruses, including the highly pathogenic Heartland virus in 2012 and Bourbon virus in 2015 (Mihindukulasuriya et al. 2009, McMullan et al. 2012, Kosoy et al. 2015). NGS also provided a platform for the investigation of tick viromes. Aside from gaining insight into viral diversity, the primary aim of tick virome studies was the discovery of novel viral pathogens. These studies were abetted by improvements in NGS, including decreased costs of sequencing, longer reads, and enhanced sequencing depth (Reuter et al. 2015). The introduction of dual labeled barcodes enabled pooling of samples, while minimizing read mis-assignments during bioinformatic analyses (MacConaill et al. 2018). NGS protocol modifications were also introduced, such as a 'viral enrichment' that included filtration and nuclease treatment prior to extraction to enhance the recovery of viral particles (Hall et al. 2014).

The first report that demonstrated the utility of NGS for tick virome discovery was published in 2014 (Tokarz et al. 2014b). It revealed the existence of a wide range of highly diverse viral sequences present in ticks within the United States. Subsequently, virome characterization analyses of a wide range of tick species from across the globe confirmed that ticks possess a very rich and diverse virome that includes representatives of *Chuviridae*, *Phenuiviridae*, *Flaviviridae*, *Orthomyxoviridae*, *Reoviridae*, *Rhabdoviridae*, *Nairoviridae*, *Nyamiviridae*, *Peribunyaviridae*, *Nairoviridae*, and *Asfarviridae*. (Tokarz et al. 2014b, Xia et al. 2015, Moutailler et al. 2016, Shi et al. 2016a, Pettersson et al. 2017, Harvey et al. 2018, Tokarz et al. 2018b, Meng et al. 2019, Sameroff et al. 2019, Gómez et al. 2020, Zhao et al. 2020, Wang et al. 2020). In *I. scapularis* alone, 18 distinct viral sequences have been identified by NGS (Tokarz et al. 2014b, Tokarz et al. 2018b, Tokarz et al. 2019).

The majority components of tick viromes are presumed to be arthropod-specific viruses and tick endosymbionts (Li et al. 2015a, Bouquet et al. 2017, Tokarz et al. 2018b, Sameroff et al. 2019). Both include viruses classified within viral orders Bunyvirales and Jingchuvirales, which are among the most frequent and abundant tick-borne viruses identified by NGS (Tokarz et al. 2014b, Li et al. 2015a, Pettersson et al. 2017, Tokarz et al. 2018b). A wide range of distinct Bunyvirales-like sequences have been found in individual tick species (Pettersson et al. 2017, Tokarz et al. 2018b). The majority of the tick-associated bunyaviruses identified by NGS consist of vertically transmitted viruses found in several *Ixodidae* tick species that lack a genomic segment encoding the glycoprotein required for receptor binding (Spiegel et al. 2016, Tokarz et al. 2018b, Vandegrift and Kapoor 2019). The Jingchuvirales order currently contains only the family *Chuviridae* and genus *Mivirus* (Li et al. 2015a). After their discovery through NGS studies of ticks, miviruses were identified in a wide range of arthropods (Tokarz et al. 2014a, Tokarz et al. 2014b, Li et al. 2015a, Pettersson et al. 2017, Harvey et al. 2018, Souza et al. 2018, Tokarz et al. 2018b, Meng et al. 2019, Sameroff et al. 2019, Temmam et al. 2019). Other, less frequently detected presumed arthropod-specific viruses and viral endosymbionts include rhabdovirus-like viruses, tetraviruses, tymoviruses, picorna/sobemo-like viruses, and pestivirus-like viruses (Tokarz et al. 2014b, Li et al. 2015a, Shi et al. 2016a, Pettersson et al. 2017, Tokarz et al. 2018b, Sameroff et al. 2019).

Outside the Americas, the primary causes of viral TBDs are Crimean Congo hemorrhagic fever virus, severe fever with thrombocytopenia syndrome virus, and tick-borne encephalitis virus (Ergönül 2006, Lindquist and Vapalahti 2008, Lei et al. 2015). In the United States, five tick-borne viruses have been implicated with human disease, including Heartland virus, Bourbon virus, Colorado tick fever virus and two distinct genotypes of Powassan virus (McLEAN and DONOHUE 1959, Telford et al. 1997, Pesko et al. 2010, McMullan et al. 2012, Kosoy et al. 2015, Yendell et al. 2015). Reports of symptomatic disease with these viruses are rare, which correlates with their infrequent detection in vector ticks. Infection rates of adult *I. scapularis* with Powassan virus are typically <5% and <2% of *A. americanum* ticks are usually infected with Heartland or Bourbon virus (Tokarz et al. 2010, 2017; Savage et al. 2013, 2016, 2017; Sanchez-Vicente et al. 2019).

Recent NGS virome studies have also identified several viruses that may require further exploration. Two virome studies identified divergent quaranjaviruses (Cholleti et al. 2018, Wille et al. 2020) classified within the family *Orthomyxoviridae*. These viruses have been isolated from the blood of children with febrile illness, with a follow-up study reporting neutralizing antibodies in about 8% of the endemic population (Mohammed et al. 1970). Another group, Jingmen tick viruses (JTVs), are genetically related to viruses belonging to the genus *Flavivirus* (Qin et al. 2014, Shi et al. 2016b). Originally identified within *Rhipicephalus microplus* (Canestrini) ticks, these viruses have since been identified in arthropods and mammals on five continents (Qin et al. 2014, Ladner et al. 2016, Emmerich et al. 2018, Souza et al. 2018, Meng et al. 2019, Pascoal et al. 2019, Sameroff et al. 2019, Vandegrift et al. 2020). Recent evidence suggests that these viruses are associated with febrile illness within China (Jia et al. 2019, Wang et al. 2019).

Eukaryotic Microbiome

The primary tick-borne eukaryotic microbes are species of apicomplexan piroplasms in the genera *Babesia* and *Theileria* that

cause babesiosis and theileriosis, respectively (Homer et al. 2000, Mans et al. 2015). Over 100 species within each genus have been described in ixodid ticks worldwide. Babesiosis is among the oldest described TBD with a long history of being a major scourge of cattle. In late 19th century, studies intended to gain insight into the cause of this disease identified *Babesia* as the very first infectious agent transmitted by an arthropod vector (Smith and Kilborne 1893). In the United States, the majority of human infections are caused by *B. microti*. Rarely have infections with other species been reported, with *B. duncani* occurring on the Pacific Coast and *B. divergens*-like species in the mid-South (Gray and Herwaldt 2019). Infections with *Theileria* spp. primarily affect horses and cattle and have no established link to human disease (Mans et al. 2015). Ticks can also harbor other apicomplexan parasites, mainly *Hepatozoon* spp., some of which may cause disease in domestic canines through ingestion of infected ticks (Allen et al. 2011, Austen et al. 2011).

Filarial nematodes have often been observed in microscopy studies of ixodid tick homogenates (Ko 1972, Londono 1976, Beaver and Burgdorfer 1984). The phylogenetic classification of the majority of these agents have remained obscure until recent PCR and NGS analyses characterized several North American species within a subgroup of *Filaroidea* (Henning et al. 2016; Cross et al. 2018; Tokarz et al. 2019, 2020). Little is currently known about the life cycles of tick-borne nematodes, although human disease has not been documented (Tokarz et al. 2020).

Other, less frequently reported tick-associated eukaryotic agents include trypanosomes and fungi (Greengarten et al. 2011, Tokarz et al. 2019). One study of *I. scapularis* identified 27 primarily entomopathogenic species of fungi at a single site (Greengarten et al. 2011). The majority of fungi are presumably acquired through environmental exposure and are likely present only on the surface of the tick. However, the presence of fungi can influence the tick virome, as certain novel viral sequences uncovered in tick shotgun sequencing studies appear to be of fungal origin (Tokarz et al. 2019).

Analyses of eukaryotic diversity in ticks have been partially impeded by the unavailability of amplicon sequencing studies analogous to bacterial 16S rRNA. Nonetheless, shotgun sequencing studies have been successful in detecting eukaryotic agents in ticks (Cross et al. 2018, Tokarz et al. 2019). These studies revealed that in comparison to viruses and bacteria, the eukaryotic component of the tick microbiome appears to be substantially less diverse.

Beyond the Microbiome

One of the primary questions that arose from the tick microbiome studies is the transmissibility of the discovered agents. This question is especially relevant to viruses, because they comprise the majority of the newly discovered and taxonomically classified tick-borne microbes. Many represent previously unknown, genetically diverse viral lineages, and therefore, no parallels can be drawn regarding their life cycle. Another challenge is that the majority appear to be refractory to common methods of tissue culture isolation, precluding studies beyond phylogenetic classification. Serologic methods could be helpful in examining the transmissibility of these agents. However, the rate of discovery of new microbes far outpaces the current capacity for such studies. NGS efforts aimed at demonstrating the presence of tick-borne agents in vertebrate hosts may be beneficial (Vandegrift et al. 2020). It has been presumed that aside from the known pathogens, the primary viral and bacterial components of the tick microbiome are non-transmissible or nonpathogenic endosymbionts. For some of these agents, studies

have begun to question this assumption (Apperson et al. 2008, Bonnet et al. 2017, Vandegrift et al. 2020). It is also plausible that endosymbionts and pathogens can directly or indirectly interact within the tick, and as a result influence pathogen transmission (Bonnet et al. 2017). Therefore, in addition to agent discovery, future studies will need to focus on the interactions between the viral, bacterial and eukaryotic components of the tick microbiome and identify new approaches to examine their role in pathogen transmission.

Conclusion

Improvements of molecular and serologic assays for detection of TBD agents have largely replaced microscopy and cultivation. PCR assays have become the mainstay for molecular detection of tick-borne agents. However, advances in sequencing technologies hold extraordinary promise. Capture sequencing, for example, uses agent-specific probes to selectively capture the template of interest before sequencing. Employment of capture probes results in an increase in yield of relevant reads compared to unbiased sequencing, and achieves a sensitivity to equivalent to real-time PCR (Briese et al. 2015, Allicock et al. 2018). Serology also has improved with the introduction of multiplex assays that employ short linear immunodominant peptides (Dessau et al. 2015, Lahey et al. 2015, Embers et al. 2016, Arumugam et al. 2019). The TBD-Serochip, for example, incorporates a wide range of specific immunodominant peptides from multiple agents. This allows simultaneous serologic detection and discrimination of antibodies to multiple TBD in a single assay (Tokarz et al. 2018a). Metabolomic analyses have identified metabolic signatures unique to early Lyme borreliosis (Molins et al. 2015, Fitzgerald et al. 2020). These studies have been extended to Southern Tick Associated Rash Illness, an illness of unknown etiology (Molins et al. 2017). Further development and refinement of these platforms will likely enable even more robust methods of diagnosis of TBD and facilitate the discovery of new agents.

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