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1 Tick-borne pathogen detection: what's new?

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Abstract

Ticks and the pathogens they transmit constitute a growing burden for human and animal health worldwide. Traditionally, tick-borne pathogen detection has been carried out using PCR-based methods that rely in known sequences for specific primers design. This approach matches with the view of a 'single-pathogen' epidemiology. Recent results, however, have stressed the importance of coinfections in pathogen ecology and evolution with impact in pathogen transmission and disease severity. New approaches, including high-throughput technologies, were then used to detect multiple pathogens, but they all need *a priori* information on the pathogens to search. Thus, those approaches are biased, limited and conceal the complexity of pathogen ecology. Currently, next generation sequencing (NGS) is applied to tick-borne pathogen detection as well as to study the interactions between pathogenic and non-pathogenic microorganisms associated to ticks, the pathobiome. The use of NGS technologies have surfaced two major points: (i) ticks are associated to complex microbial communities and (ii) the relation between pathogens and microbiota is bidirectional. Notably, a new challenge emerges from NGS experiments, data analysis. Discovering associations among a high number of microorganisms is not trivial and therefore most current NGS studies report lists of microorganisms without further insights. An alternative to this is the combination of NGS with analytical tools such as network analysis to unravel the structure of microbial communities associated to ticks in different ecosystems.

Keywords: ticks, pathogen detection, next generation sequencing, network analysis

Tick-borne pathogens: "One health" concern

Ticks are hematophagous ectoparasites of vertebrates and approximately 10% of the 900 currently known tick species are of significant medical or veterinary importance. Besides causing direct damage associated with blood feeding and in some cases through the excretion of toxins within their saliva [1], the main relevance of ticks lies in the wide variety of pathogens they can transmit, including bacteria, viruses, protozoa and [2-4]. After hatching from the eggs, the life cycle of ticks includes three developmental stages (larvae, nymphs and adults) that in most cases (i.e. three-host ticks) feed on different hosts. Potentially, while feeding on a host, each of these stages can transmit and acquire new pathogens [5]. Thus, ticks are 'hubs' in pathogen's circulation cycles. Major tick-borne pathogens are transmitted by hard ticks (Ixodidae) and include *Anaplasma phagocytophilum*, *Borrelia burgdorferi sensu lato*, Crimean-Congo hemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), *Rickettsia* spp. and *Babesia* spp., [4]. These pathogens cause the most prevalent tick-borne diseases such as human granulocytic anaplasmosis (*A. phagocytophilum*), Lyme diseases (*B. burgdorferi*), Crimean-Congo hemorrhagic fever (CCHFV), tick-borne encephalitis (TBEV), spotted fever (*Rickettsia* spp.) and babesiosis (*Babesia* spp.). Other major human pathogens may occasionally be transmitted by ticks, including *Francisella tularensis* and *Coxiella burnetii*. Importantly, the circulation of tick-borne pathogens in nature involves wildlife and livestock which pose a twofold risk for animal and human health [6].

Single-pathogen models

Our current experimental and theoretical models of pathogen transmission by ticks are limited because they frequently include single pathogen species [7-9]. Despite their limits, single-

pathogen models have allowed for the systematic discovery of tick-borne microorganisms with pathogenic effects in humans and livestock. Detection and identification of single pathogens is not technically demanding and relies mostly on PCR [10, 11]. After amplification of some taxonomically relevant genes by PCR, sequencing is followed by BLAST search and, in some cases, phylogenetic analysis for pathogen classification [10-13]. A major limitation of this approach is that it is extremely biased towards known pathogens as species-specific primers for PCR are designed based on known sequences. As a consequence, pathogen detection within the same geographic region will be strongly influenced by particular research interests. Another limitation of one-pathogen models is that they do not explain the impact of coinfections on pathogen transmission, on the spread of diseases and on the clinical presentation.

Why coinfections are important?

Coinfections, when multiple pathogen species coexist within an individual, are very common in nature [14, 15] and are a major public health concern. Coinfections occur in humans, such as by the malaria parasite, *Plasmodium* [16], in the setting of sexually-transmitted infections or mixed abdominal infections. It may also occur in a wide range of other organisms, from bacteria infected by a mixture of bacteriophages [17] to plants [18] and animals [19]. When pathogens share a host, they can interact, with consequences for individual pathogen fitness [14, 20]. Individual pathogens can adapt and increase their fitness in response to coinfections if pathogens facilitate each other's establishment [21]. Alternatively, it has been shown that individual infection rates can be reduced if pathogens directly compete via resources or toxin-production, or indirectly interact via host immune-mediation, whereby one pathogen primes the host immune response against the other (e.g. cross-reactivity) [15]. Epidemiological

studies in natural populations have provided compelling evidence that within-host interactions are so strong that the dynamics of one pathogen, within a host and within a host population, cannot be understood without knowledge of other pathogens [14, 22].

Probably, one of the best studied examples of tick-borne pathogen coinfection is that of *A. phagocytophilum* and *B. burgdorferi*. These two pathogens have been systematically reported in the literature [23], as well as in clinical cases of humans [24] as occurring together more often than expected by chance. In USA, coinfection with *B. burgdorferi* and *A. phagocytophilum* have been reported in *Ixodes scapularis* [23], as well as in humans [24] and wild animal hosts [25]. Tick infection and colonization by *A. phagocytophilum* and *B. burgdorferi* occurs firstly in the tick gut cells and subsequently in other tissues, including the salivary glands from where transmission occurs during feeding. Thus, these pathogens coexist and potentially interact within the same tissues for long periods of time. Empirical work has shown that coinfection with these two pathogens can enhance pathogen colonization in tick larvae [26], and significantly increase the potential for the spread of Lyme disease. Coinfections also elicit different immune system responses within mice hosts – the antibody response to *A. phagocytophilum* was decreased during coinfection, but antibodies produced in response to *B. burgdorferi* increased in coinfecting mice [27] – as well as pathological processes – *A. phagocytophilum*-infected neutrophils enhance the transmigration of *B. burgdorferi* across the human blood brain barrier [28]. All this suggests that coinfection has a major impact on the fitness, transmission and pathology of these two pathogens.

In another study, natural populations of field voles (n=5981), *Microtus agrestis*, were followed for 6 years and coinfections with cowpox virus, *Bartonella* spp., *A. phagocytophilum* and *Babesia microti* were recorded [14]. This impressive field experiment revealed that except for cowpox, infection with other parasite species explained more

variation in infection risk than factors related to exposure risk and host condition, such as age and season [14]. Interestingly, voles with ongoing *A. phagocytophilum* infections were less likely to become infected with *B. microti*, but risk was not reduced in animals that had recently cleared an infection [14].

Currently, coinfections are routinely included in tick-borne pathogen screenings [29-31]. Thus, the Swiss national center for tick-borne diseases sequentially tested the same 8'000 batch of ticks for the presence of the agent of tick-borne encephalitis [32], for chlamydiae [13] as well as *Anaplasma* and *Coxiella* (Pilloux et al, unpublished). Not only different pathogen species were found coinfecting ticks and hosts, but also coinfections with multiple strains of the same pathogen have been reported [33, 34]. Detection of coinfections can be achieved following standard PCR or more demanding technologies such as microfluidic high-throughput real-time PCR. This nanotechnology is a powerful tool capable of performing parallel real-time PCRs using 96x96 chips resulting in 9216 individual reactions in one run [35]. Recently, Michelet and colleagues [29] applied this technology for a large (n=7050 ticks) and rapid screening of tick-borne pathogens in *Ixodes ricinus*, the most common tick in Europe. These authors successfully detected expected pathogens (*B. burgdorferi* sensu lato, *A. phagocytophilum*, *Rickettsia helvetica*, *Candidatus Neorickettsia mikurensis*, *Babesia divergens*, *Babesia venatorum*), as well as unexpected pathogens (*Borrelia miyamotoi*), and rare (*Bartonella henselae*) pathogens in France, Denmark, and the Netherlands [29]. This technology can be easily adapted to detect 'single pathogens' or 'multiple pathogens' infections. However, despite the leap of this technology compared to standard PCR, both have the same limitation, this is, to be highly biased towards known pathogens as species-specific primers have to be designed based on known sequences.

Despite this review focuses on microorganisms detected in ticks, it is noteworthy that coinfections with different tick-borne pathogens are frequently reported in humans [36-38]. Strikingly, the majority of patients with chronic Lyme disease reported at least one coinfection with another tick-borne pathogen. In particular, 32.3% reported laboratory confirmed diagnosis with *Babesia*, 28.3% with *Bartonella* (note that only *B. henselae* is suspected to be transmitted by ticks), 14.5% with *Ehrlichia*, 4.8% with *Anaplasma*, 5.6% with Rocky Mountain spotted fever (caused by *Rickettsia rickettsii*), and 0.8% with tularemia [36]. An interesting example is that of the human coinfection of *B. burgdorferi* with *B. microti* in the United States [38]. The emergence of *B. microti* has become difficult to explain because this pathogen has a low ecological fitness characterized by poor transmission from *Peromyscus leucopus* mice to larval ticks and poor transstadial transmission from larvae to nymphs [38]. Interestingly, recent studies show that human babesiosis is emerging in areas endemic for Lyme disease. The current hypothesis is that *B. burgdorferi* increases *B. microti* transmission from *P. leucopus* mice to ticks [38]. The current model that explains the epidemiology of *B. microti* in the United States demonstrates that the emergence of tick-borne infections should be studied within realistic epidemiological and ecological contexts. Selected examples of relevant tick-borne pathogen coinfections are provided in Table 1.

Understanding the tick pathobiome

Recent advances in next generation sequencing (NGS) technologies applied to explore the tick microbiome revealed an astonishing diversity of microorganisms associated to these arthropods [39-43]. These studies using NGS have shown that specific tick-borne pathogens are frequently found together with other pathogens, symbionts and commensals. This was described as a technology-driven revolution of tick-borne pathogen's vision and the concept

160 of pathobiome was proposed [3]. This theoretical framework recognizes that the pathogen is
 161 integrated within its abiotic and biotic (i.e. including other pathogens, commensals and
 162 symbionts) environment [3]. Different NGS technologies have been utilized to define the
 163 microbiomes of various tick species: Sanger sequencing of full-length 16S rDNA, 454-
 164 pyrosequencing, Ion torrent, or Illumina-based sequencing of 16S rDNA hypervariable
 165 regions, as well as a whole genome shotgun [41]. A major strength of NGS compared to PCR-
 166 based approaches is that NGS is not biased towards the detection of specific microorganisms.

167 There is functional evidence that the relation between pathogen and microbiome is
 168 bidirectional. For example, in the tick *I. scapularis*, the gut microbiota composition influences
 169 *B. burgdorferi* colonization of tick guts [40]. A tick gut microbiota composed by high
 170 abundance of bacteria of the genera *Rickettsia*, *Thioclava*, and *Delftia*, and low abundance of
 171 bacteria of the genera *Aquabacterium*, *Brevibacterium*, and *Novosphingobium* did not favor *B.*
 172 *burgdorferi* colonization of tick guts [40]. This microbiota composition, which was recovered
 173 from ticks reared and maintained under “sterile” conditions, decreased the expression of the
 174 transcription factor signal transducer and activator of transcription (STAT). Lower STAT
 175 expression correlated with diminished expression of peritrophin, a component of the tick
 176 peritrophic matrix, which in turn decreased the ability of *B. burgdorferi* to colonize the gut
 177 epithelium [40]. Another interesting example showed that tick colonization by *A.*
 178 *phagocytophilum* perturbs the tick gut microbiota by decreasing the relative abundance of
 179 *Enterococcus* and *Rickettsia* whereas increasing the abundance of *Pseudomonas* [42].
 180 *Anaplasma phagocytophilum* induces *I. scapularis* to express antifreeze glycoprotein, which
 181 encodes a protein that modulates the peritrophic matrix and binds Gram-positive bacteria
 182 decreasing their ability for biofilm formation [42]. Thus, by inducing antifreeze glycoprotein
 183 expression, *A. phagocytophilum* modifies tick microbiota and tick peritrophic matrix which

184 may jeopardize *B. burgdorferi* colonization [40, 42]. While these studies provided some
185 functional basis of pathogen-microbiome interactions, a major challenge remains to
186 understand the pathobiome at the bacterial community level. The analysis of bacterial
187 communities as a whole may be challenging and specific analytical tools are needed to this
188 aim.

189 Network analysis is a suitable tool that has been used to unravel complex microbial
190 communities as those present in soil [44], water [45] or animal microbiota [46, 47]. Recently,
191 ecological networks methodology was applied to unravel the complex interactions between
192 ticks, their vertebrate hosts and pathogens in the western Palearctic [6]. Using data mining,
193 more than 14,000 interactions were quantified among ticks, vertebrates, and pathogens in the
194 western Palearctic [6]. The use of this approach, allowed concluding that ticks and vertebrates
195 interact along the shared environmental gradient, while pathogens are linked to groups of
196 phylogenetically close reservoirs [6]. Another report using networks methodology revealed a
197 prominent role for birds in the dissemination of *B. burgdorferi* and *A. phagocytophilum*, with
198 little contribution to the possible dissemination of other tick-borne pathogens [48]. This was
199 in agreement with the fact that *B. burgdorferi* (*s.l.*) complex circulation is supported by a
200 highly redundant network where few host genera have high centrality values (i.e. high relative
201 importance for pathogen circulation) [49]. NGS projects, such as those performed currently to
202 study the ecology of tick-associated microorganisms [50, 51], generate large data sets that can
203 be combined with networks analysis.

204 Finally, NGS studies have revealed that 'single or multiple-pathogens infection' are both
205 idealized scenarios that do not reflect a more complex reality where 'pathogen transmission'
206 appear to be a limited conception of a broader phenomenon, i.e. microorganisms; including
207 pathogens, symbionts and commensals; migrate across biological systems. In fact, several

symbionts can transmit horizontally when their hosts interact through mating, feeding or egg laying [52, 56]. For example, the male-killing heritable symbiont *Arsenophonus nasoniae* is transmitted horizontally when their parasitoid wasp host share oviposition patches with uninfected conspecifics, a phenomenon called superparasitism [55, 56]. Interestingly, *Candidatus Midichloria mitochondrii*, a tick endosymbiont, was proposed to be transmitted both vertically and horizontally [57, 58]. Further NGS studies should evaluate the hypothesis of the transmission of microbial communities in vector-host systems (i.e. between ticks and between ticks and hosts).

Concluding remarks

In the last twenty years, tick-borne pathogen detection have improved dramatically from ‘single’ and ‘multiple’ pathogens detection to the elucidation of the pathobiome. The ‘single pathogen’ view is still widely used and indeed is a necessary ‘reduction’ that should be integrated to the studies addressing the complexity of the pathobiome. Combining NGS projects with network analysis will provide new insights into the structure of microbial communities associated to ticks and their impact on pathogen circulation.

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380

381 **Table 1. Selected examples of ticks co-infection significantly modifying the biology of**
 382 **another microbe co-occurring in ticks.**

383	<u>Co-infections</u>	<u>Effect</u>
384	<i>Anaplasma/Borrelia</i>	Decreased antibody response towards
385		<i>A. phagocytophilum</i>
386	<i>Borrelia/Anaplasma</i>	Increased transmigration of <i>B. burgdorferi</i>
387		across the human blood brain barrier
388	<u><i>Borrelia/Babesia microti</i></u>	<u>Increased transmission from mice to ticks</u>