Tick-borne diseases and co-infection: Current considerations

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Abstract:

Over recent years, a multitude of pathogens have been reported to be tick-borne. Given this, it is unsurprising that these might co-exist within the same tick, however our understanding of the interactions of these agents both within the tick and vertebrate host remains poorly defined. Despite the rich diversity of ticks, relatively few regularly feed on humans, 12 belonging to argasid and 20 ixodid species, and literature on co-infection is only available for a few of these species. The interplay of various pathogen combinations upon the vertebrate host and tick vector represents a current knowledge gap. The impact of co-infection in humans further extends into diagnostic challenges arising when multiple pathogens are encountered and we have little current data upon which to make therapeutic recommendations for those with multiple infections. Despite these short-comings, there is now increasing recognition of co-infections and current research efforts are providing valuable insights into dynamics of pathogen interactions whether they facilitate or antagonise each other. Much of this existing data is focussed upon simultaneous infection, however the consequences of sequential infection also need to be addressed. To this end, it is timely to review current understanding and highlight those areas still to address.

Introduction:

Tick-bites are commonplace but are limited to a relatively small number of species that regularly bite humans (Estrada-Peña and Jongejan, 1999). The health implications of tick-borne diseases in Europe and beyond are only recently becoming appreciated. Anthropogenic influences, variation of faunal composition of vertebrates, social-recreational changes and climatic trends have all contributed towards changes in the distribution of ticks and patterns of risk for human tick bites (Medlock et al., 2013). This is coupled with our increasing awareness of new and emerging tick-borne pathogens, and growing recognition of the significance of established tick-borne pathogens for human health. It is only recently that consideration of multiple tick-borne pathogens has been questioned and our diagnostic capability advanced to facilitate multiplex assays to screen for multiple agents. Tick-borne co-infections are the result of infection with genetically distinct pathogens that might be closely related such as variants within the same species through to diversely different pathogens such as parasites and bacteria or viruses. Ticks can acquire multiple pathogenic species (such as parasites, bacteria or viruses), through systemic transmission during blood feeding on their different vertebrate hosts, or through co-feeding, whereby the host serves as a “bridge” enabling uninfected ticks to acquire infection through spatiotemporal proximity with infected ticks feeding on the same host (Voordouw, 2015). Importantly, the latter of these mechanisms can facilitate transmission to uninfected ticks feeding upon vertebrate hosts considered immune through prior exposure or even enable transmission between ticks feeding upon a host unable to support systemic infection (Voordouw, 2015), further enhancing the potential to acquire multiple pathogens (Jacquet et al., 2016). Regarding co-infection, as the actual tick that transmitted

infection is rarely available, we argue that evidence of tick bite serves only to demonstrate exposure to tick-infested habitats and that co-infection might follow the bite of one or more ticks either simultaneously or following sequential transmission events. From the clinical perspective, we define tick-borne co-infection as that acquired by transmission from one or multiple ticks either following a single exposure or multiple sequential exposures.

Co-infections do not just present diagnostic challenges, but the pathogens might behave synergistically, indifferently or antagonistically within their respective hosts, thus modulating disease severity (Belongia, 2002; Diuk-Wasser et al., 2016; States et al., 2017). Furthermore, presence of other microbes can act as a driving force facilitating emergence or successful co-existence of pathogens within their ecological niche (Diuk-Wasser et al., 2016; States et al., 2017). Though still in its infancy, we explore co-infection within ticks and their subsequent hosts, providing insights into the challenges and gaps that need to be addressed in this largely over-looked field.

Co-Infections Within Ticks:

Co-operative interactions between microbes

Several studies have reported findings of multiple pathogens in ticks, some focussing upon selected pathogens, whereas others have used microbiome approaches. These reports are often limited by inclusion of both questing and feeding ticks, with the latter potentially reporting presence of agents that cannot be further transmitted by the next developmental stage of that tick. Further limitations of studies assessing interactions of pathogenic species within ticks have combined microbes transmitted in different ways, such as those predominantly using horizontal

transmission with those with considerable vertical transmission, complicating interpretation of findings. Finally, it is difficult to conclude whether observed findings can be attributed entirely to the tick and its pathogenic content or are influenced by vertebrate hosts of the previous blood meal. Mathematical analytical methods to evaluate whether synergistic or antagonistic relationships exist between different pathogens were proposed by Ginsberg whereby positive or negative interactions could be compared using observed co-infection rates with the expected prevalence based upon individual pathogens (Ginsberg, 2008).

In a study addressing co-infection of *Borrelia burgdorferi* sensu lato (s.l.) and *Rickettsia* species involving 5079 predominantly questing *Ixodes ricinus* ticks, it was found that significantly more co-infections were evident than predicted by individual infection rates. Interestingly, this was driven by infection rates in nymphs alone which showed a 48% increase of coinfection (453/3572 compared with the predicted value of 308/3572). Overall 12.3% (626/5079) of ticks were co-infected with both *B. burgdorferi* s.l. and *Rickettsia* spp. compared with the predicted co-infection rate of 9% (457/5079) (Raulf et al., 2018). Single infections for *B. burgdorferi* s.l. were 25.6% (1301/5079 and 35.2% (1786/5079) for *Rickettsia* spp. respectively (Raulf et al., 2018). Others have similarly observed higher mixed pathogen rates (mostly *Borrelia* and *Rickettsia* or *Borrelia* and *Candidatus* Neoehrlichia mikurensis) amongst questing nymphs (7.2% of 457 nymphs) rather than questing adults (5.2% of 77 adults) (Raileanu et al., 2017). The relative lack of co-infection contribution from adult ticks was interpreted as a potential detrimental influence of co-infection on the ability of ticks to successfully moult to adult stages. This might have been in consequence of the higher bacterial loads observed amongst the 173/1365 adult

ticks assayed revealing a 2-fold increase in microbial load for co-infected ticks above singly infected ticks for *Borrelia* and a 6.12-fold increase for those infected with *Rickettsia* (Raulf et al., 2018).

A large study from the United States that analysed 16,080 ticks, largely removed from humans, reported that 88% (139/158) of ticks positive for pathogens were co-infected, representing 0.86% of all ticks tested. The most frequently encountered combination amongst *I. scapularis* being *Anaplasma phagocytophilum* and *B. burgdorferi* sensu stricto (s.s.) within 1.7% (79/4671) ticks tested, followed by *Babesia microti* and *B. burgdorferi* s.s. 0.8% (36/4671) ticks tested. Furthermore, seven of these ticks revealed triple infection (0.1% 7/4671) (Nieto et al., 2018). Given the predominance of co-infection studies reporting both *A. phagocytophilum* and *B. burgdorferi* s.l., a meta-analysis of 4978 questing adult ticks belonging to four species within the *I. ricinus* complex (*I. pacificus; I. persulcatus; I. ricinus; I. scapularis*), was undertaken to assess potential synergies or interference based upon data derived from single infections (Civitello et al., 2010). Of these ticks, 31.6% (1573 ticks) carried *B. burgdorferi* s.l., whereas 14.4% (716 ticks) were positive for *A. phagocytophilum*. Co-infection with both agents was reported in 5.4% (268 ticks). Analysis comparing observed levels of co-infection with those predicted by chance revealed 44% of tick populations studied (8 of 18 reports), demonstrated considerable deviations from predicted levels of co-infection. Interestingly, this varied with tick species, with *I. ricinus* largely showing facilitation, whilst *I. persulcatus* showed less co-infection than expected. *Ixodes scapularis* ticks varied in their ability to support both pathogens, suggesting more complex microbial interactions influencing the ability to host both microbes (Civitello et al., 2010).

144

145 Rickettsial co-infection with various other pathogens however appeared
146 commonplace amongst 170 *Amblyomma variegatum* ticks feeding on cattle from
147 Cote d'Ivoire, of which 90% contained *R. africae* found in combination with *Coxiella*
148 *burnetii; Anaplasma centrale; Anaplasma marginale*; novel *Borrelia* spp. and
149 *Ehrlichia* spp. (Ehounoud et al., 2016).

150

151 Presence of co-infection has been further corroborated using metagenomics
152 approaches to assess the pathogen content of *I. ricinus* ticks, with frequent detection
153 of co-infection between *B. burgdorferi* s.l. and *Rickettsia* spp; *Rickettsia* spp and
154 *Anaplasma* spp. and triple infection of *B. burgdorferi* s.l., *Rickettsia* spp. and
155 *Anaplasma* spp. One tick studied had a dual rickettsial infection (*Rickettsia* sp. and a
156 *Rickettsiella*) (Jose Oteo Revuelta personal communication). Similarly, metagenomic
157 studies of *I. scapularis* have demonstrated co-infection in 19% of ticks (38/197), with
158 16% being dual infections (32/197) and 3% triple infections (Tokarz et al., 2019), but
159 without notable correlations. Triple infection of *I. scapularis* with *B. burgdorferi* s.s.*;*
160 *B. microti;* and *A. phagocytophilum* has also been reported by others (Cross et al.,
161 2018). This latter study also noted considerable correlations between presence of
162 South Bay virus, a probable viral mutualistic symbiont, and either *Rickettsia* spp. or
163 *B. burgdorferi* s.s. (Cross et al., 2018), though this observation was not confirmed by
164 others (Tokarz et al., 2019). On a cautionary note, species belonging to the genera
165 of *Coxiella, Francisella* and *Rickettsia* are remarkably commonplace in multiple tick
166 species, most probably serving as a symbiont within ticks (Abreu et al., 2019; Bonnet
167 et al., 2017; Chicana et al., 2019; Clay et al., 2008; Lim et al., 2019). These

168 symbionts are evolutionarily close to many tick-borne pathogens, and some have
169 been incriminated as potential vertebrate pathogens (Abreu et al., 2019).
170
171 What is becoming increasingly apparent is the complexity of microbial interactions
172 within the tick that involves not only pathogenic members, but also non-pathogenic
173 members of the microbial community derived from the tick's environment, host
174 encounters and parental lineages, that can either facilitate or antagonise the
175 presence of pathogenic counterparts (Bonnet et al., 2017). Microbial flora has been
176 reported to vary between sympatric tick species, life stages and host ranges
177 (Chicana et al., 2019; Hawlena et al., 2013) and is influenced within a single tick
178 species by feeding upon different hosts (Landesman et al., 2019). Indeed, given
179 these new insights, interpretation of vector competence and the interaction of co-
180 infecting pathogens is likely to demand more comprehensive microbiome evaluation
181 in order to provide meaningful interpretation (Eisen, 2019).
182
183 Benefits to ticks
184 In more general terms, experimental data suggest that the presence of borreliae
185 within a tick confers a survival advantage for ticks exposed to adverse conditions,
186 with the suggestion that ticks are better able to survive and were more aggressive,
187 therefore better suited to endure the cycle of questing in the vegetation that exhaust
188 tick energy reserves (Herrmann and Gern 2010).
189
190 Evidence suggests that some members of the relapsing fever group of borreliae
191 arose from a symbiotic spirochaete of an arthropod (Estrada-Peña et al., 2018a).
192 This mimics the evolutionary picture proposed for the coxiellae (Bonnet et al., 2017;

193 Duron et al., 2015). Furthermore, many tick endosymbionts are phylogenetically close
194 to species considered as pathogens in their accidental human hosts such as
195 members of the rickettsiae (Duh et al., 2010) and coxiellae (Bonnet et al., 2017; Duron
196 et al., 2015; Moutailler et al., 2016).
197
198 Microbial modulation of ticks
199 The ability of pathogens to modulate the tick physiology to facilitate its survival and
200 proliferation has been explored using transcriptomic and proteomic analysis. Such
201 studies have shown that *A. phagocytophilum* is able to manipulate the tick
202 environment enhancing its own survival (Abraham et al., 2017; Bonnet et al., 2017;
203 Estrada-Peña et al., 2018b). A complete overview of the effects of the infection by *A.*
204 *phagocytophilum* on the tick's metabolome has been recently undertaken (Estrada-
205 Peña et al., 2018b) resulting in a complete rewiring of the metabolic functions within
206 the tick. Furthermore, the location within the tick is correlated with different forms of
207 the pathogen, with the intracellular replicative forms of this pathogen located within
208 the tick gut, whilst the infective condensed form is observed within the tick salivary
209 gland (Ayllón et al., 2015). Unsurprisingly, modification of the tick internal
210 environment is likely to enable other pathogens to survive and be co-transmitted
211 alongside *A. phagocytophilum*. Notably, ticks have been reported to harbour both *A.*
212 *phagocytophilum* and *Borrelia* species (Ekner et al., 2011; Jahfari et al., 2016;
213 Lindblom et al., 2013), but the efficacy of this relationship can vary with tick species
214 and other factors impacting upon the tick microbiome (Civitello et al., 2010; Clay and
215 Fuqua, 2010). These findings also highlight the need to understand not just the
216 interplay of ticks and their pathogens, but also to understand the specific interactions
217 of the microbes within different tick organs that enable their co-transmission.

Further studies on *A. phagocytophilum* have demonstrated the infection of ticks stimulates the over-representation of tick heat shock proteins (Busby et al., 2012), a group of chaperones that protect the tick against the stress. This has not yet been studied for *Borrelia*-tick interactions to our knowledge, but comparison of infected versus uninfected tick cell lines could provide a clue about potential manipulative behaviour of *Borrelia* upon tick metabolic pathways.

Microbial interference

When looking at rickettsial interactions, it has been observed that presence of endosymbiont species can exclude the presence of the human pathogen *Rickettsia rickettsii*, the cause of rocky mountain spotted fever (Baldridge et al., 2004; Clay and Fuqua, 2010; Macaluso et al., 2002; Moutailler et al., 2016). The precise mechanisms of this exclusion remain elusive, but there is an obvious potential for biological intervention to reduce the prevalence of *R. rickettsii* among its *Dermacentor andersoni* tick vector. Furthermore, *R. rickettsii* has a deleterious effect upon its tick vector with a vertical transmission rate of just 39% coupled with reduced survival and fecundity when compared to uninfected counterparts (Niebylski et al., 1999). Experimental studies using capillary inoculation of *Dermacentor variabilis* ticks with either established infections with *R. montana* or *R. rhipicephali*, demonstrated exclusion of transovarial transmission of the second rickettsial species in reciprocal infections (Macaluso et al., 2002). In Europe, where different tick species are sympatric and likely to share vertebrate host species such as *D. reticulatus* and *I. ricinus*, the rickettsial species carried by each tick appears to segregate according to tick species, with *R. slovaca* and *R. raoultii* present in *D.*

reticulatus, whilst *R. helvetica* and *R. monacensis* were found within *I. ricinus* ticks (Švehlová et al., 2014). This might reflect tick-specific competence for transovarial transmission or a highly specific tick-microbial specificity, possibly mediated through the tick haemocytes (Hernandez et al., 2019), or tick innate immune-related proteins (Chmelař et al., 2016; Hernandez et al., 2019), or through *Rickettsia*-mediated exclusion of subsequent rickettsial species (Macaluso et al., 2002).

Recent findings have demonstrated how certain pathogens can influence the tick gut microbiota, with colonisation of the tick gut by *A. phagocytophilum* exerting an interference with biofilm formation, mediated by the tick glycoprotein (iafgp). This resulted in a noticeable change in microbial abundance such as reduction in both *Enterococcus* and *Rickettsia*, whereas numbers of *Pseudomonas* increased (Abraham et al., 2017). Deeper exploration of tick holobionts is likely to help us decipher the complex interactions of different ticks and their microbial assemblages.

Vertebrate Host Influences Upon Tick-Pathogen Interactions:

Vertebrate host-mediated bias of co-infecting pathogens

Efficacy of vertebrates to serve as reservoirs is based upon both the duration of infectivity combined with the mortality rate of this host, thus its ability to serve as a host for subsequent ticks. This has been explored extensively for members of *B. burgdorferi* s.l. Within this complex of species, a distinct variation has been observed regarding vertebrate vector competence, believed to be driven by the ability of particular genospecies to withstand the complement of their vertebrate host species (Bhide et al., 2005; Kurtenbach et al., 2002a, 1998b). This complement susceptibility underpins the ecological niche utilised by different borrelial species, with *B. garinii,*

and *B. valaisiana* having a reservoir in avian hosts, whilst other species have various rodents as their vertebrate reservoir (Hanincova et al., 2003; Hanincová et al., 2003; Kurtenbach et al., 1998a). This divergent host association will have obvious impact upon co-infections acquired by ticks, with evidence from nymphal *I. ricinus* ticks collected in Switzerland (n=7400; 20.5% [1520] with single *Borrelia* genospecies; 2.8% [211] with double; and 0.13% [10] with triple infections), demonstrating positive correlations (facilitation) amongst co-infections compatible with the same vertebrate host (Herrmann et al., 2013), thus showing positive or negative pairwise interactions between genospecies (those genospecies surviving in avian hosts, or alternatively those found in rodents). Conversely, when genospecies adapted to divergent host species (rodent vs avian) were assessed, frequency of dual infection was less than expected based upon single genospecies infection data, thus showing negative correlation (Herrmann et al., 2013).

Reservoir status of various vertebrate species was clarified by Gern and co-authors who although were focussed upon *Borrelia* transmission alone, promoted the value of xenodiagnoses as the gold standard to determine ability to serve as a reservoir host (Gern et al., 1998). Successful co-feeding transmission can occur to non-infected ticks clustered into preferred host feeding sites such as around ears of mammals or the bill area of birds, and in the absence of detectable systemic infection. This clustering is further enhanced by production of aggregation pheromones by some ticks (Randolph et al., 1996). Collectively, these approaches provide us with the tools to enable evaluation of the ability of vertebrates to influence co-infection of ticks.

293 Defining reservoir host competency based upon evidence of systemic infection has
294 been increasingly challenged. Many species deemed refractory to pathogens may
295 indeed contribute towards the ecology of tick-borne pathogens through tick co-
296 feeding as opposed to systemic routes (Belli et al., 2017; Ogden et al., 1997;
297 Randolph et al., 1996; States et al., 2017). Evidence supporting potential non-
298 systemic transmission was derived from the finding of larval ticks attached to birds
299 that were harbouring the rodent associated *B. afzelii* (Heylen et al., 2016),
300 furthermore, there appears to be considerable exposure of birds to nymphal ticks
301 infected with the rodent associated *B. afzelii* (Kurtenbach et al., 1998a; Lommano et
302 al., 2014; Sparagano et al., 2015). *In vivo* experimental competence studies further
303 substantiated the very limited ability of birds to facilitate tick infection with *B. afzelii*
304 though co-feeding (Heylen et al., 2016; Kurtenbach et al., 2002b) . Interestingly,
305 Heylen and co-researchers attempted to cultivate the *B. afzelii* from the nymphs
306 used to challenge birds, without success despite their continued positivity by PCR.
307 On a cautionary note, this suggests that literature reports of molecular detection of
308 both *B. afzelii* and *B. garinii* in infected nymphs might not be detecting viable
309 organisms (Heylen et al., 2016), or that ticks may have fed on multiple hosts (Moran
310 Cadenas et al., 2007; Pichon et al., 2005).
311
312 Segregation of different borrelial genospecies within ticks that have fed upon
313 different hosts (avian or rodent), impacts upon ecology of borreliae within their
314 ecosystems, but may not represent the entire story. Complement incompatibility is
315 undoubtedly important in selecting the *B. burgdorferi* s.l. species able to cause
316 systemic infection, but does not extend to those that might transfer to naïve ticks
317 through co-feeding (see Fig. 1) (Belli et al., 2017; Heylen et al., 2017; Hua et al.,

2003; Ogden et al., 1997; Patrican, 1997; Pérez et al., 2011; Richter et al., 2002; Sato and Nakao, 1997; Voordouw, 2015). Evidence for the importance of co-feeding in hosts unable to support systemic infection was described for sheep that successfully facilitated transmission of borreliae in absence of detectable bacteraemia (Ogden et al., 1997).

Studies of natural reservoir species such as the bank vole, *Myodes glareolus*, suggest that co-infection is more frequent (54.3%) than single infection with *B. afzelii* strains (Andersson et al., 2013). This study revealed a mean of 2.58 strains per host with some hosting up to six strains. It was postulated that this might serve as a facilitation factor enabling multiple strains to survive the host immune defence better than homogeneous infection: the species would persist at the cost of a heterogeneity at the bacterial strain level (cf. *supra*). Further benefit may be derived from variable adaptation to their vertebrate host such as through tissue tropism (see multiple niche polymorphism below). However, the outcome upon human health of co-infections by multiple strains in terms of which strain is more perdurable and its pathogenic potential, remains to be elucidated.

Where highly related borrelial species or even members of the same species have been compared, we again see differences in the successful competence of transmission between vertebrate and tick (see Fig. 1). Interestingly, in a study comparing different isolates of *B. burgdorferi* s.s., it was observed that homologous re-infection had no negative impact upon transmissibility to xenodiagnostic ticks, however prior infection with one strain negatively impacted upon the successful transmission of the second strain. Interestingly the converse was not observed, thus

343 demonstrating asymmetric competition (Rynkiewicz et al., 2017). This begs the
344 question of why the strain that demonstrated reduced transmission has remained
345 within this shared ecological niche? Presumably this strain had other capabilities that
346 counterbalanced its susceptibility to the dominant strain in this experimental model
347 that only investigated one species of rodent (*Peromyscus leucopus*) (Rynkiewicz et
348 al., 2017). This further serves to highlight how sequential co-infections can have very
349 different outcomes to those observed during simultaneous infection.
350
351 Transmission efficacy studies of *Borrelia* both in Europe (Tonetti et al., 2015) and
352 using *Peromyscus leucopus* for *B. burgdorferi* s.s. transmission within *I. scapularis* in
353 the United States, have provided valuable insights (States et al., 2017). This latter
354 study compared two different OspC strains of *B. burgdorferi* s.s. (OspC types C and
355 E, with differing abilities for rodent infection (Antonara et al., 2010; Strle et al., 2011).
356 The work by States and co-authors suggested that both co-feeding transmission
357 occurred between simultaneously feeding ticks, but also localised transmission
358 occurred with uninfected ticks acquiring infection up to 10 days post infected tick
359 detachment, provided an important transmission route to infect naïve ticks in the
360 absence of systemic infection. The overall contribution of these transmission routes
361 to sustaining tick infection varied depending upon the strains assessed and the
362 synchrony of nymphal and larval tick feeding times (States et al., 2017).
363
364 The OspC types of *B. burgdorferi* s.s. have been correlated with human virulence
365 potential (Dykhuizen et al., 2008; Lagal et al., 2006, 2003; Seinost et al., 1999;
366 Wormser et al., 2008), but also differ regarding their ability to persist through
367 systemic infection of rodent reservoirs and to adapt to their tick vectors (Derdáková

et al., 2004; Hanincová et al., 2008; Jacquet et al., 2016; Rynkiewicz et al., 2017; Tonetti et al., 2015). Indeed, this strain diversity linked to OspC types appears to play a pivotal role in the survival of *B. burgdorferi* s.l. within its varied ecological environments through multiple niche polymorphism (Brisson et al., 2012; Brisson and Dykhuizen, 2004; Vuong et al., 2014). These observations have not been confirmed by others with a study of *B. afzelii* in Sweden failing to demonstrate significant host correlation of OspC types with reservoir rodent species (Råberg et al., 2017).

As seen among the borreliae, specific host clusters have been described for different *A. phagocytophilum* strains or ecotypes associated with vertebrate host species and were reported in different species of ticks (*I. ricinus, I. hexagonus, I. frontalis* and *I. trianguliceps*), even in sympatry (Jahfari et al., 2014). The ability of each of these tick species to serve as vectors requires transmission studies. Some of these ecotypes have only been reported in voles and rodents, others in birds, but not in human infection underscoring the need to understand the transmission dynamics at an ecotype level (Jahfari et al., 2014; Majazki et al., 2013). Consequently, molecular strain typing is a priority to decipher the ecological transmission dynamics and risks for co-infection by different *Anaplasma* strains.

Pathogen facilitation within the vertebrate host

Occurrence of co-infection of *B. burgdorferi* s.s. and *B. microti* appears frequently in the North-eastern United States (Diuk-Wasser et al., 2016). Where this occurs, current evidence suggests that in rodents transmission of *B. microti* is enhanced within hosts previously infected with *B. burgdorferi* s.s.(Dunn et al., 2014). It is postulated that this may result from immune-modulation impairing the splenic Th1

393 response essential for clearance of *B. microti*. Furthermore, it is suggested that
394 presence of *B. burgdorferi* s.s. might serve as a driver facilitating emergence of
395 *Babesia* into new areas as seen in North-eastern regions of the United States (Dunn
396 et al., 2014; Vaumourin et al., 2015). Others have failed to show increased co-
397 infection of *B. burgdorferi* s.s. and *B. microti* above that predicted by each pathogen
398 individually (Egizi et al., 2018). This synergy effect does appear to vary with the
399 OspC phenotype of *B. burgdorferi* s.s. with the more invasive strains showing
400 enhanced facilitation of *B. microti* compared to their less virulent counterparts (Dunn
401 et al., 2014).
402
403 Similarly, assessment of tick-borne infections amongst rodents captured from
404 Hokkaido, Japan, revealed levels of co-infection above the predicted expected
405 prevalence for either *B. microti* and *Ca.* N. mikurensis alone (Moustafa et al., 2016).
406 This study also reported that co-infection appeared more frequently in male rodents
407 (15.9% vs 18.3% for *B. microti*; 8.2% vs 16.9% *Ca*. N. mikurensis; 3.4% vs 4.8%
408 *Anaplasma* sp.; 0% vs 2.2% *Ehrlichia muris*; female:male respectively) and in the
409 late summer months (highest infection rates found in September). Others have found
410 high frequencies of dual infections of *Ca.* N. mikurensis with *B. afzelii* in their natural
411 host, the bank vole, *M. glareolus* (Andersson et al., 2014). It was hypothesised that
412 immunosuppression caused by one pathogen might facilitate infection by the
413 second. This remains to be tested, however no difference was observed between
414 infection intensity between either single or co-infections.
415
416 *In vivo* studies for predicting consequences of co-infection for humans

Various *in vivo* laboratory studies have used different mouse lineages to explore the effects of co-infection of *Borrelia* and *A. phagocytophilum* or *Babesia*, with a view to extrapolating this to human risk (Bhanot and Parveen, 2019; Coleman et al., 2005; Holden et al., 2005; Moro et al., 2002; Thomas et al., 2001). These studies have shown contradictory findings, some suggesting increased severity (Moro et al., 2002), whilst others report indifference in the clinical course following dual infection (Coleman et al., 2005). Some have reported a skew with advantages gleaned by one pathogen, but reduced impact of the other, such as seen with co-infection of *B. burgdorferi* s.s. and *B. microti* whereby the clinical signs attributed to *Borrelia* are exacerbated whilst the parasitaemia burden for *B. microti* was reduced during co-infection (Bhanot and Parveen, 2019). In part, variation has been attributed through use of different murine strains and pathogen genotypes that might vary in their virulence potential. These studies have been limited to comparing single infection with dual infection given simultaneously to a naïve host and consequently have not addressed the impact of sequential infection. This is an important limitation of these studies that overlook the potential interactions of the initial infection that might alter the subsequent dynamics of the newly incoming pathogen, for example for closely related pathogens, having to deal with a mature and potentially cross-protective immune response against the initial infecting strain, or for unrelated pathogens having the benefits from a more polarised vertebrate host immune response (Vaumourin et al., 2015). Either way, it is challenging to extrapolate these observations to provide corroborating evidence for observed human pathology.

Co-Infection in the Human Host:

Often, and indeed in much of the above review, co-infection is considered as a potential result from a single co-infected tick. It must be remembered that tick bites are not always noted, particularly those from immature stages, thus having been in a tick-infested area could be sufficient for the acquisition of multiple tick-borne pathogens even if they were not derived from a single tick feeding. Furthermore, exposure to a tick-infested habitat is often regular through environmental proximity, recreational or occupational activities, consequently transmission events might be sequential rather than simultaneous. Indeed, from this perspective one should query the relevance of the foregoing studies determining the prevalence of multiple pathogens within individual ticks as this is unlikely to relate to the risk for human co-infection beyond driving numbers of pathogens within each ecological niche and thus likelihood of encounter.

The dynamic interactions between tick-borne pathogens and subsequent human infections are complex and poorly understood. Studies to date have largely targeted the pathogens deemed to have greatest human impact and consequently have focussed on *B. burgdorferi* s. l., *Babesia* spp., *A. phagocytophilum,* tick-borne encephalitis virus (TBEv) and other tick-borne flaviviruses/phleboviruses (Garcia-Monco and Benach, 2019; Kemenesi and Bányai, 2019; Kurhade et al., 2018; Ruzek et al., 2019; Schroeder et al., 2017). A plethora of studies have assessed seropositivity against multiple pathogen combinations to draw their conclusions (Caulfield and Pritt, 2015; Curcio et al., 2016; Hilton et al., 1999; Lantos, 2015; Wass et al., 2018). It must be remembered that these were often conducted in highly endemic regions, where significant percentages of healthy controls showed seroreactivity against organisms such as *A. phagocytophilum*, thus potentially

limiting the impact of these studies. Molecular investigations of *Ixodes* ticks have shown that in the United States, co-infection with *B. burgdorferi* s.l., *A. phagocytophilum* and *B. microti/divergens* occurs in 1% to 28%, whilst in Europe this is seen amongst <1% to13% of ticks sampled (Swanson et al., 2006).

The contribution of *A. phagocytophilum* to human co-infection is further complicated by the presence of different ecotypes with marked differences in host species susceptibilities (Jahfari et al., 2014; Majazki et al., 2013; Stuen et al., 2013). Indeed, this might be a factor responsible for much of the asymptomatic seroconversion of humans to *A. phagocytophilum* often reported from European studies. Unsurprisingly, significantly more seropositives for human anaplasmosis were detected amongst individuals with other tick-borne infection with reports of 13% amongst those with *B. burgdorferi* s.l. infection and 20% of those with TBE (Pusterla et al., 1998).

One of the few studies specifically addressing co-infection of human subjects reported that a third of 92 human biting ticks were positive for *Borrelia* species, but also contained an unrelated pathogenic species (Jahfari et al., 2016). Furthermore, analysis of post-tick bite or erythema migrans bloods revealed that 2.5% (16/626) had positive PCR for pathogens including *Ca.* N. mikurensis, *A. phagocytophilum, B. divergens, B. miyamotoi,* and *B. burgdorferi* s.l. Nevertheless, additional clinical signs related to these other pathogens were not recorded during a three-month follow-up period (Jahfari et al., 2016). Others report clinical cases of co-infection of *B. afzelii* and TBEv, but without significant worsening of consequences (Boyer et al., 2018). Furthermore, as infection with Lyme-associated *Borrelia* appears more

frequent than infection with other agents, and in cases presenting with erythema migrans, empiric treatment with doxycycline would be given in the absence of confirmatory tests, thus concomitant tick-borne pathogens would often go unnoticed. With the exception of erythema migrans, many other clinical signs associated with tick-borne infection give only limited clinical insights into the likely causative agents, necessitating laboratory diagnostics to give a more informed interpretation. Use of blood smears, full blood counts and liver function tests have proven useful, particularly for anaplasmosis and babesiosis. It is noteworthy that in endemic regions, 20% of patients with Lyme borreliosis additionally have babesiosis, whereas 25% of those with babesiosis are concomitantly infected with *B. burgdorferi* s.l. (Parveen and Bhanot, 2019). Serology has been used extensively but can be imprecise particularly in highly endemic areas where persisting titres through prior exposure complicate interpretation. Serological interpretation can be further challenged given the close genetic relationship between some tick-borne pathogens and non-pathogenic symbionts present in tick (Bonnet et al., 2017), potentially accounting for the considerable serological reactivity to multiple tick-borne agents in sera from patients with Lyme borreliosis (Garg et al., 2018). Multiplex molecular diagnostics similarly play a major role in detection of co-infection (Moutailler et al., 2016; Tokarz et al., 2017), but these too have limitations such as the duration of positive findings and the optimal sample type to evaluate for all tick-borne pathogens.

Importantly, concurrent infection with multiple tick-borne agents has been reported by several groups to have a greater clinical severity than would be expected with either infection in isolation (Diuk-Wasser et al., 2016; Swanson et al., 2006). An *in*

vitro study of human brain microvascular endothelial cells co-infected with both *B. burgdorferi* s.s. and *A. phagocytophilum*, noted reduction of the transendothelial electrical resistance coupled with modulation of the production of cytokines, chemokines and tumour necrosis factor that would collectively influence vascular permeability and the inflammatory response to infection (Grab et al., 2007). The cumulative effects could enhance the ability of pathogens to disseminate thus impacting upon clinical severity.

The drivers underpinning the changing patterns of tick-borne infections have in part been discussed above, but largely arise from alteration in the natural equilibrium by combinations of biotic and abiotic factors including human behaviour, climatic changes, different land use, urbanisation and discovery of new pathogens. Absence of data regarding risk areas, lack of appropriate multiplex diagnostics, poor comprehension of clinical consequences and particular risk populations, collectively make understanding of co-infections as a human health priority challenging.

Impact:

With some 1.1 million estimated tick bites during 2007 being reported in the Netherlands alone and prospective follow up of a cohort of 293 individuals bitten by 314 ticks with a *B. burgdorferi* s.l. prevalence of 29.3%, resulted in 2.6% of those available at follow-up developing erythema migrans within three months of the reported tick bites (Hofhuis et al., 2015, 2013). To assess the impact of co-infections there is a need to deploy surveillance of exposed humans over time, but this is often complicated by the prompt removal of ticks feeding on humans, thus reducing likelihood of successful pathogen transmission coupled with the ethical

considerations necessitating prompt therapeutic intervention. Tick surveillance has provided useful information regarding co-circulation of pathogens within different tick vectors and mapping the distribution of tick species in relation to climate and land use factors such as fragmentation. Such tick prevalence data is undoubtedly useful but fails to reflect the dynamic interplay of co-infecting pathogens within the vertebrate host, making the need for robust models to the disease progression due to co-infecting pathogens a priority. Data generated from these sources can be compiled within mathematical models to predict areas of disease emergence or changing disease patterns. Statutory surveillance for human tick-borne infections has been restricted to tularaemia and Crimean-Congo haemorrhagic fever, though some countries now also report Lyme borreliosis and TBE. This is often complicated by differing case definitions that often fail to account for the possibility of co-infections. Alternative approaches to assess co-infection risks include use of sentinel animals, however this is costly and can result in over-estimation of risk.

Despite availability of a vaccine, the annual notification of TBE in the EU during 2012-2015 fluctuated between 0.41 cases per 100,000 population in 2015 and 0.65/100,000 in 2013, associated with a case fatality rate of 0.5% (HZ personal communication). Of these, 2000-3000 were officially reported to ECDC (ECDC TESSy data, in 2012 -2015). Tenfold more cases of Lyme borreliosis occur, and in Germany 9/100,000 per annum required inpatient hospital care (although a diagnostic breakdown was not provided, more than half of the adult patients were admitted to neurological wards), with an associated direct economic impact of 23 million Euros and a further 7 million Euros of indirect impact through loss of productivity (Lohr et al., 2015). Worryingly, incidence of these infections appears to

566 be increasing and several tick vectors and their associated pathogens have been
567 noted to be expanding their endemic regions (Jaenson et al., 2012; Ogden, 2017;
568 Sykes and Makiello, 2017). Reports from New Jersey, USA, suggest a four-fold
569 increase in human babesiosis whereas anaplasmosis has increased seven-fold
570 when comparing the average annual incidence from 2002-2005 with that reported
571 between 2006-2015 (Egizi et al., 2018). Assessment of risk and modelling studies for
572 tick-borne pathogens have been based upon single tick-pathogen relationships,
573 often assuming all genotypes to have similar pathogenic potential. Our expanding
574 knowledge highlights the limits of such generalisations and does not accommodate
575 the nuances that result from co-infection whereby certain pathogens might facilitate
576 the presence of others in either host or vector, or indeed the influences of the
577 individual tick microbiome. Furthermore, the impact of co-infection upon
578 pathogenesis remains to be elucidated. This necessitates diagnostic algorithms that
579 should consider co-infections in common practice rather than being a clinical oddity.
580 Well-designed and controlled studies with sufficient statistical power are needed to
581 answer some of the key questions listed in Table 1 that represents substantial gaps
582 in our current understanding of co-infections.
583
584 Concluding Remarks:
585 We are beginning to gain insights into the complex pathogen-tick-host dynamics
586 depicted in Fig. 1 and the underpinning factors that might influence changes in
587 microbial prevalence. Co-existence of different microbes within these diverse
588 environments adds an additional layer of complexity to the relationship dynamics
589 with some exploiting this to enhance their transmission, whilst others suffer
590 detrimental consequences. Our discussions above are often based upon

591 generalities, assuming all members of a microbial group are equal and indeed, that
592 all categories of the vectors and their hosts are again equivalent, which is probably
593 an overly simplistic interpretation. Despite this, some of the examples given in our
594 discussion serve as a starting point from which models can emerge to help us
595 understand the interplay within the host-microbial-vector paradigm. These may need
596 to be modified to accommodate our increasing knowledge such as appreciation of
597 the contribution of co-feeding transmission, whether simultaneous or sequential, and
598 the synergistic facilitation or exclusion influences of one pathogen, or indeed the
599 tick's own microbiome, might exert on the survival of another. This is particularly
600 important when we consider the public health implications of co-infection whereby
601 transmission can be influenced by the presence of pathogen combinations, clinical
602 course altered, and therapeutic interventions more challenging.
603
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Fig. 1: Dynamic interactions of ticks, their pathogens and hosts.

Table 1: Key questions and knowledge gaps regarding co-infections.