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COEXISTENCE OF EMERGING BACTERIAL PATHOGENS IN *IXODES RICINUS* TICKS IN SERBIA[1]

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Summary:

The list of tick-borne pathogens is long, varied and includes viruses, bacteria, protozoa and nematodes. As all of these agents can exist in ticks, their co-infections have been previously reported. We studied co-infections of emerging bacterial pathogens (*Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Francisella tularensis*) in *Ixodes ricinus* ticks in Serbia. Using PCR technique, we detected species-specific sequences, *rrf-rrl* rDNA intergenic spacer for *B. burgdorferi* s.l., *p44/msp2* paralogs for *A. phagocytophilum*, and the 17 kDa lipoprotein gene, TUL4, for *F. tularensis*, respectively, in total DNA extracted from the ticks. Common infections with more than one pathogen were detected in 42 (28.8 %) of 146 infected *I. ricinus* ticks. Co-infections with two pathogens were present in 39 (26.7 %) of infected ticks. Simultaneous presence of *A. phagocytophilum* and different genospecies of *B. burgdorferi* s.l. complex was recorded in 16 ticks, co-infection with different *B. burgdorferi* s. l. genospecies was found in 15 ticks and eight ticks harbored mixed infections with *F. tularensis* and *B. burgdorferi* s.l. genospecies. Less common were triple pathogen species infections, detected in three ticks, one infected with *A. phagocytophilum* / *B. burgdorferi* s.s. / *B. lusitaniae* and two infected with *F. tularensis* / *B. burgdorferi* s.s. / *B. lusitaniae*. No mixed infections of *A. phagocytophilum* and *F. tularensis* were detected.

KEY WORDS: Borrelia burgdorferi s.l., *Anaplasma phagocytophilum*, *Francisella tularensis*, co-infection, tick, Serbia.

Résumé :

La liste des pathogènes portés par les tiques est longue et variée, et inclue des virus, des bactéries, des protozoaires et des nématodes. Des co-infections par ces divers pathogènes ont été précédemment rapportées. Nous avons étudié les co-infections bactériennes par Borrelia burgdorferi *sensu lato,* Anaplasma phagocytophilum *et* Francisella tularensis *chez la tique* Ixodes ricinus *en Serbie. Par PCR, nous avons détecté des séquences spécifiques d'espèce de ces trois bactéries dans de l'ADN total extrait de la tique. Des infections avec plus d'un pathogène ont été détectées chez 42 (28,8 %) des 146* I. ricinus *infectés. Les co-infections avec deux pathogènes étaient présentes dans 39 (26,7 %) de ces 146 tiques infectées. La présence simultanée d'*A. phagocytophilum *et de différents génotypes du complexe* B. burgdorferi *s.l. a été observée chez 16 tiques ; la co-infection par différents génotypes de* B. burgdorferi *s.l. chez 15 tiques, et huit présentaient une infection mixte par* F. tularensis *et* B. burgdorferi *s.l. Des infections triples, plus rares, ont été détectées chez trois tiques, une infectée par* A. phagocytophilum, B. burgdorferi *s.s. et* B. lusitaniae, *et les deux autres par* F. tularensis, B. burgdorferi *s.s. et* B. lusitaniae. *Aucune infection associant* A. phagocytophilum *et* F. tularensis *n'a été observée.*

MOTS CLÉS : Borrelia burgdorferi *s.l.*, Anaplasma phagocytophilum, Francisella tularensis, *co-infection, tique, Serbie.*

Recognition of ticks and tick-borne diseases as an emerging problem in temperate regions during last decades coincides with increasing human-induced climatic and urban changes in the environment. Among all European tick species, *Ixodes ricinus* is the most widely distributed. Its wide ecological amplitude and very aggressive and undiscriminating behavior during questing for a host make it one of the main transmitters of causative agents of infectious diseases among humans. The species *I. ricinus* is usually predominant among ticks originating from Serbia and is one of the most widely distributed (Milutinović, 1992; Milutinović & Radulović, 2002). Fifteen pathogens were identified as emerging in Europe from 2000 to 2006, including bacterial pathogens transmitted by *Ixodes ricinus* species: *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Francisella tularensis*. As all of these agents can exist in ticks, their co-infections have been previously reported (Fingerle *et al.*, 1999; Christova *et al.*, 2001; Stańczak *et al.*, 2004; Wojcik-Fatla *et al.*, 2009). Ticks can become infected with multiple pathogens after a single blood meal from a co-infected host or by feeding on single infected hosts during sequential life stages (Levin & Fish, 2000). Co-infection with tick-associated pathogens has the potential to modulate their transmission dynamics at multiple points

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in the transmission chain. This includes alterations in the efficiency of transmission from host to tick or vice versa, cooperative or competitive pathogen interactions and increasing or decreasing disease severity among hosts (Thompson *et al.*, 2001). Human co-infection with tick-borne pathogens can occur after attachment of a single tick infected with multiple pathogens or from concurrent single pathogen tick attachments. Any disease developing as a result of tick bite should be regarded as a potentially mixed infection.

European studies of pathogen co-infections in *I. ricinus* ticks predominantly involved *B. burgdorferi* s.l., *A. phagocytophilum* and *Babesia microti* / *Babesia divergens*. The prevalence of dual pathogens differed depending on the geographic site of tick sampling and the methodology, with the highest occurrence reported in Bulgaria (13.4 %) (Christova *et al.*, 2001), Poland (10.6 %) (Stanczák *et al.*, 2004) and Italy (8.1 %) (Cinco *et al.*, 1997). Investigations of the simultaneous presence of *F. tularensis* with other pathogenic species are very limited. Christova & Gladnishka, (2005) detected co-infections of *F. tularensis* and *B. burgdorferi* s.l. in 4.7 % of rodents from Bulgaria, while records from ticks are lacking.

The aim of our study was to determine the presence and prevalence of *B. burgdorferi* s.l. genospecies, *A. phagocytophilum* and *F. tularensis* co-infections in *I. ricinus* ticks from Serbia. To our knowledge, this is the first attempt to investigate simultaneous presence of these pathogenic groups in *I. ricinus* ticks.

MATERIAL AND METHODS

Study area and tick collection

Host-seeking adult *I. ricinus* were collected at 18 localities (Fig. 1 and Table I) from three regions (Vojvodina, Belgrade area and Inner Serbia) in Serbia by dragging white flannel flags over vegetation in 2001, 2003, and 2004. Collected ticks were stored in 70 % ethanol until assay.

Extraction of DNA

DNA was isolated from whole tick tissues using the QuickGene-800 Nucleic-acid Isolation System with the QuickGene DNA tissue kit (Fujifilm Co., Tokyo, Japan). The quality of extracted DNA was evaluated by PCR targeted at the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes according to a previously described method (Fukunaga *et al.* 2000). Primer set 5.8S F3/1 (5'-GGG TCG ATG AAG AAC GCA GCC AGC-3') and 28S R1/1 (5'-TTC AGG GGG TTG TCT CGC CTG ATG-3') was used. The PCR conditions were one cycle consisting of 5 min of denaturation at 94 °C, followed by 35 cycles consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, and 2 min of extension at 72 °C.

Detection and genotyping of *B. burgdorferi* s.l. species

DNAs prepared from tick tissue were subjected to *rrf-rrl* rDNA intergenic spacer PCR by a method described previously (Masuzawa *et al.*, 1996). Primers corresponding to the 3' end of 5S rDNA (*rrf*) (RIS1; 5'-CTG CGA GTT CGC GGG AGA-3' and RIS3; 5'-GGA GAG TAG GTT ATT GCC AGG-3') and the 5' end of 23S rDNA (*rrl*) (RIS2; 5'-TCC TAG GCA TTC ACC ATA-3' and RIS4; 5'-GAC TCT TAT TAC TTT GAC C-3') were synthesized by a custom oligonucleotide synthesis service (Invitrogen, Tokyo, Japan). Primer sets RIS1 and RIS2, and RIS3 and RIS4 were used for first-step and nested-PCR, respectively, under the previously described PCR conditions (Masuzawa *et al.*, 1996). Determination of *B. burgdorferi* sensu lato genospecies was performed by the restriction fragment length polymorphism (RFLP) technique, using restriction enzymes *Mse*I and *Dra*I as previously described (Günter *et al.*, 2003; Postic *et al.*, 1994).

Detection of *A. phagocytophilum* specific DNA *p44/msp2*

For detection of *A. phagocytophilum* DNA, nested-PCR using primers designed on the basis of the highly conserved region of *p44/msp2* paralogs of p3726F [5'-GCT AAG GAG TTA GCT TAT GA-3'], p3761F [5'-CTG CTC T(T/G)G CCA A(A/G)A CCT C-3'], p4183R [5'-CAA TAG T(C/T)T TAG CTA GTA ACC-3'], and p4257R [5'-AGA AGA TCA TAA CAA GCA TTG-3'] was conducted

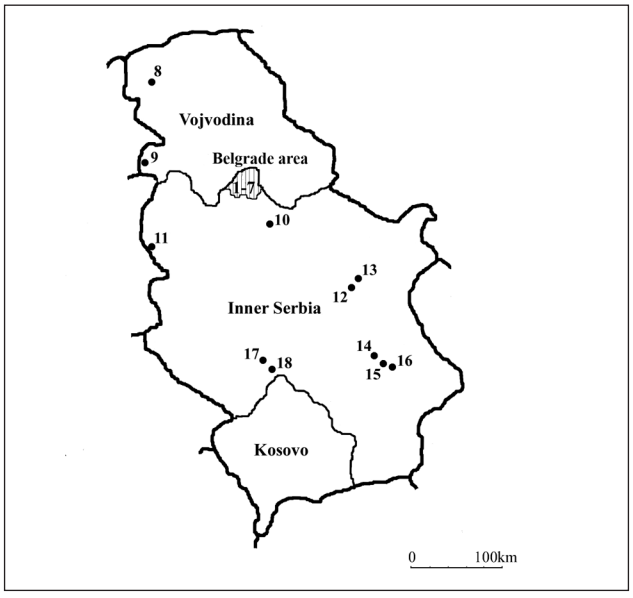

Fig. 1. – Map of Serbia with tick collection sites (Sites 1 to 7 – Belgrade area; 8, 9 – Vojvodina; 10 to 18 – Inner Serbia)

(Ohashi *et al.*, 2005). The PCR conditions were one cycle consisting of 5 min of denaturation at 94 ºC, followed by 35 cycles consisting of 0.5 min of denaturation at 94 ºC, 1 min of annealing at 58 ºC, and 1 min of extension at 72 ºC. The second step of the nested-PCR was conducted with 1 µl of the first-step PCR product as template DNA under the same conditions as described above.

DETECTION OF *F. TULARENSIS*-SPECIFIC DNA ENCODING 17 KDA LIPOPROTEIN (TUL4)

For detection of *F. tularensis* by PCR, the primer set TUL4-435 (5'-GCT GTA TCA TCA TTT AAT AAA CTG CTG-3') and TUL4-863 (5'-TTG GGA AGC TTG TAT CAT GGC ACT-3') was used as reported previously (Sjöstedt *et al.*, 1997). The PCR conditions were one cycle consisting of 5 min of denaturation at 94 ºC, followed by 35 cycles consisting of 0.5 min of denaturation at 94 ºC, 0.5 min of annealing at 55 ºC, and 0.5 min of extension at 72 ºC.

NUCLEOTIDE SEQUENCE ANALYSIS

All *Anaplasma* and *Francisella* and some of *Borrelia burgdorferi* s.l. positive samples were sequenced afther cloning. The DNA cycle sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. DNA sequences were determined using the Applied Biosystems 3130-genetic analyzer.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The sequences of *B. burgdorferi* s.l., *A. phagocytophilum* and *F. tularensis* detected in this study are available in the GenBank database under accession numbers AB437927 to AB437932 (*flab* gen of *Borrelia burgdorferi* s.l.), AB295961 to AB296065 (*p44* paralogs of *A. phagocytophilum*) and AB262327 to AB262329 (TUL4 gene of *F. tularensis*).

STATISTICAL ANALYSIS

The number of ticks positive and negative for each pathogen was compiled in 2x2 contingency tables and chi-square test was used to assess significance. To quantify the degree of departure of the number of mixed infections from independence, index of co-infection (Ic) was used (Ginsberg, 2008).

RESULTS

Different genospecies of *B. burgdorferi* s.l. complex were present in 140 of the 287 analyzed ticks – *B. burgdorferi* s.s. in 39 (13.6 %), *B. afzelii* in 22 (7.7 %), *B. garinii* in 14 (4.9 %), *B. lusitaniae* in 54 (18.8 %) and *B. valaisiana* in 11 (3.8 %) of analyzed ticks. *Anaplasma phagocytophilum* was detected in 40 (13.9 %) and *F. tularensis* in 11 (3.8 %) ticks (Table I).

Infections with more than one pathogen were detected in 42 (28.8 %) of 146 infected *I. ricinus* ticks. Co-infections with two pathogens were present in 39 (26.7 %) of infected ticks. Simultaneous presence of *A. phagocytophilum* and different genospecies of *B. burgdorferi* s.l. complex was recorded in 16 ticks, two different *B. burgdorferi* s.l. genospecies were present in 15 ticks and eight ticks harbored mixed infections with *F. tularensis* and *B. burgdorferi* s.l. genospecies. Less common were triple pathogen species infections, detected in three ticks, one infected with *A. phagocytophilum* / *B. burgdorferi* s.s. / *B. lusitaniae* and two infected with *F. tularensis* / *B. burgdorferi* s.s. / *B. lusitaniae* (Table II). No mixed infections with *A. phagocytophilum* and *F. tularensis* were detected.

Statistical analysis of all co-infected individuals is presented in Table III. The number of mixed infections differed significantly ($p < 0.05$) from expectation due to chance alone in four cases (*B.burgdorferii* s.s. / *B. lusitaniae*, *B. garinii* / *B. valaisiana*, *F. tularensis* / *B. burgdorferi* s.s. and *F. tularensis* / *B. lusitaniae*) and Ic was positive in all four cases, indicating positive interactions among these pathogens within a tick. The significance was the highest in cases of *F. tularensis* co-infections with *B. burgdorferi* s.l. genospecies. Out of 11 *Francisella* positive ticks, 10 were co-infected with *B. burgdorferi* s.l. genospecies, suggesting that infection with one of these pathogens also helped another

Region	No. of ticks examined	Pathogen						
		Borrelia burgdorferi s.l.					*Anaplasma phagocytophilum*	*Francisella tularensis*
		B.b.s.s	*B.a.*	*B.g.*	*B.l.*	*B.v.*		
Vojvodina	17	2	0	1	0	0	3	0
Belgrade area	121	18	12	3	37	3	14	7
Inner Serbia	149	19	10	10	17	8	23	4
Total	287	39	22	14	54	11	40	11

Table I. – Prevalence of *B. burgdorferi* s.l., *A. phagocytophilum*, and *F. tularensis* in adult *I. ricinus* ticks from Serbia.

Type of co-infection	Region			Total
	Vojvodina	Belgrade region	Inner Serbia	
B.b.s.s. / B.l.	0	4 (3.3 %)	6 (4 %)	10 (3.5 %)
B.b.s.s. / B.a.	0	0	1 (0.7 %)	1 (0.3 %)
B.g. / B.v.	0	0	2 (1.3 %)	2 (0.7 %)
B.a. / B.l.	0	1 (0.8 %)	0	1 (0.3 %)
B.a. / B.v.	0	0	1 (0.7 %)	1 (0.3 %)
A.ph. / B.a.	0	3 (2.5 %)	3 (2 %)	6 (2.1 %)
A.ph. / B.l.	0	2 (1.6 %)	2 (1.3 %)	4 (1.4 %)
A.ph. / B.b.s.s.	1 (5.9 %)	0	3 (2 %)	4 (1.4 %)
A.ph. / B.g.	0	1 (0.8 %)	0	1 (0.3 %)
A.ph./B.v.	0	1 (0.8 %)	0	1 (0.3 %)
A.ph. / B.b.s.s. / B.l.	0	1 (0.8 %)	0	1 (0.3 %)
F. tul./ B.b.s.s.	0	1 (0.8 %)	2 (1.3 %)	3 (1 %)
F. tul. /B.l.	0	4 (3.3 %)	1 (0.7 %)	5 (1.7 %)
F. tul. / B.b.s.s. / B.l.	0	2 (1.6 %)	0	2 (0.7 %)

Table II. – Type and number of co-infections in adult *I. ricinus* ticks from Serbia.

Type of co-infection	p	Ic
B.b.s.s. / B.l.	**0.0126**	**+ 7.077**
B.g. / B.v.	**0.0367**	**+ 6.363**
B.a. / B.l.	0.0747	– 4.186
B.a. / B.v.	0.8562	+ 0.490
B.b.s.s. / B.a.	0.1977	– 3.316
A.ph. / B.a.	0.0602	+ 5.239
A.ph. / B.l.	0.1241	– 3.918
A.ph. / B.b.s.s.	0.8285	– 0.589
A.ph. / B.g.	0.4517	– 1.795
A.ph. / B.v.	0.6360	– 1.066
F. tul. / B.b.s.s.	**0.0017**	**+ 7.789**
F. tul. / B.l.	**0.0001**	**+ 8.501**

p = probability from chi-square test, Ic = index of co-infection – [types of co-infection with statistical significance ($p < 0.05$) are presented in bold].

Table III. – Statistical analysis of *B. burgdorferi* s.l., *A. phagocytophilum*, and *F. tularensis* co-infection rates.

bacterium to infect a tick. In two more cases (*B. afzelii / B. lusitaniae* and *A. phagocytophilum / B. afzelii*) differences between observed and expected ratio of co-infections were close to level of significance. Index of co-infection (Ic) for *A. phagocytophilum* and *B. afzelii* was positive, while mixed infection analysis of *B. afzelii* and *B. lusitaniae* showed negative Ic, indicating some level of negative interaction between pathogens.

DISCUSSION

Our results demonstrate presence of different *B. burgdorferi* s.l., *A. phagocytophilum* and *F. tularesis* co-infections in 14.6 % of analyzed ticks from Serbia and in 28.8 % of those infected. Among them, 1 % of analyzed ticks harbored triple pathogen infections. To our knowledge, this is the first study dealing with the simultaneous presence of these three emerging pathogens in *I. ricinus* ticks, so no relevant data were found for adequate comparison. European studies of different pathogen co-infections in *I. ricinus* ticks showed prevalence rates up to 13.4 % (Christova *et al.*, 2001), while simultaneous presence of three pathogen species is rarely detected and usually with a prevalence < 1 % (Swanson *et al.*, 2006).

Borrelia burgdorferi s.l. genospecies co-infections were detected in 5.2 % of analyzed ticks in Serbia. This type of co-infection is common in areas where *B. burgdorferi* s.l. diversity is present, but with lower prevalence rates comparing with those of infection with a single genospecies (Hubálek & Halouzka, 1997; Kirstein *et al.*, 1997; Schouls *et al.*, 1999; Christova *et al.*, 2001; Kurtenbach *et al.*, 2001; Hildebrandt *et al.*, 2003; Koči *et al.*, 2007). It seems that the distribution of different *B. burgdorferi* s.l. co-infection in individual ticks is rather a result of aggregation or segregation among genospecies, than an independent phenomenon. Thus, *B. garinii / B. valaisiana* mixed infection is noted more frequently than expected according to the presence of individual genospecies, while simultaneous presence of *B. garinii* and *B. afzelii* occurs less frequently than expected (Kurtenbach *et al.*, 2001). The most dominant type of co-infection detected in our research was *B. burgdorferi* s.s / *B. lusitaniae*, with a positive index of co-infection and a significantly higher prevalence rate than expected. This type of mixed infection is recorded sporadically in Europe (Postic *et al.*, 1997; Christova *et al.*, 2001) but never with such dominancy over other co-infections. *Borrelia burgdorferi* s.s. strains are adapted to both mammalian and avian hosts. Concerning *B. lusitaniae*, recent studies suggest that competent reservoirs of this genospecies are lizards (Dsouli *et al.*, 2006), but Poupon *et al.* (2006) found that migratory birds may be reservoir

hosts for this genospecies. According to simultaneous presence of these two genospecies in *I. ricinus* ticks in Serbia, we can assume that enzoonotic cycles of *B. burgdorferi* s.s. and *B. lusitaniae* are overlapping at some point, leading to positive interactions among them within a tick. *Borrelia garinii / B. valaisiana* co-infection rate was significantly higher than expected, while no *B. afzelii / B. garinii* and *B. burgdorferi* s.s. / *B. valaisiana* mixed infections were detected. These results are in agreement with the findings of other authors (Kurtenbach *et al.*, 2001; Rauter & Hartung, 2005).

Simultaneous presence of two different emerging pathogens was detected in 8.4 % of analyzed ticks in Serbia (*B. burgdorferi* s.l. / *A. phagocytophilum* – 5.6 % and *B. burgdorferi* s.l. / *F. tularensis* – 2.8 %). In comparison, most European *B. burgdorferi* s.l. / *A. phagocytophilum* co-infection studies (Baumgarten *et al.*, 1999; Fingerle *et al.*, 1999; Hildebrandt *et al.*, 2003; Schouls *et al.*, 1999; Skotarczak *et al.*, 2003) showed lower prevalence rates, while higher prevalence rates were detected in Bulgaria (13.4 %) (Christova *et al.*, 2001), Italy (8.1 %) (Cinco *et al.*, 1997) and Slovakia (7.5 %) (Derdáková *et al.*, 2003). *Borrelia afzelii* was most frequently detected simultaneously with *A. phagocytophilum*, with a difference between the observed and expected ratio of co-infections close to the level of significance and a positive index of co-infection. This type of mixed infection was frequently observed by other authors (Koči *et al.*, 2007; Skarphédinsson *et al.*, 2007; Masuzawa *et al.*, 2008).

Francisella tularensis was detected in mixed infections with two most dominant *B. burgdorferi* s.l. genospecies – *B. burgdorferi* s.s. and *B. lusitaniae*. The frequency of both types of co-infections was highly significant with a positive index of co-infections indicating positive interactions between these pathogens within a tick. There are few data about *F. tularensis* co-infections with other pathogens. Christova & Gladnishka (2005) detect this type of mixed infection in 4.7 % of analyzed rodents in Bulgaria, while no data on co-infection presence in *I. ricinus* ticks has been noted so far.

The possibility of multiple infection in humans due to a single tick bite was proven (Levin & Fish, 2000), and there is evidence of concurrent multiple human infections caused by tick-borne agents (Magnerelli *et al.*, 1998; Steere *et al.*, 2003; Lotrič *et al.*, 2009). The epidemiology of tick-borne co-infections is mainly derived from serological studies of patients with suspected or confirmed Lyme disease. Prior studies have suggested that human co-infections with *A. phagocytophilum* and *F. tularensis* are common in Europe (Christova & Dumler, 1999; Santino *et al.*, 2004; Tomasiewicz *et al.*, 2004) with a prevalence up to 17 % in Switzerland (Dumler *et al.*, 1997). The data on the prevalence of co-infections in humans are lacking in Serbia. Based on the presence of mixed infections in *I. ricinus* ticks we can presume the presence of concomitant infections with these emerging pathogens in the resident population.

Anaplasma phagocytophilum and *F. tularensis* are known as human pathogens causing human granulocytic anaplasmosis (HGA) and tularemia, respectively (Rikihisa, 1991; Oyston, 2004). All five detected *B. burgdorferi* s.l. genospecies are known to be pathogenic for humans (Van Dam *et al.*, 1993; Collares-Pereira *et al.*, 2004; Diza *et al.*, 2004). Clinically, tick-borne mixed infections proceed more severely than the corresponding diseases caused by a single agent, implying the significance of a comprehensive approach to prevention, diagnosis and treatment. In this respect, our findings of ticks simultaneously infected with three different pathogenic species (one with *A. phagocytophilum / B. burgdorferi* s.s. / *B. lusitaniae* and two with *F. tularensis / B. burgdorferi* s.s. / *B. lusitaniae*) are of great concern. The presented results will provide a basis for prevention and control of these tick-borne diseases in Serbia. Though we point to genetic heterogeneity among co-infecting pathogens, the epidemiology and natural history of co-infections have not been fully understood, and more detailed study in this field is needed.

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