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Prevalence of *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato, *Rickettsia* spp. and *Babesia* spp. in cattle serum and questing ticks from Belgium

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ABSTRACT

Background: Anaplasmosis, borreliosis, rickettsiosis and babesiosis are tick-borne diseases of medical, veterinary and economic importance. In Belgium, little is known on the prevalence of these diseases in animals and previous screenings relate only to targeted geographic regions, clinical cases or a limited number of tested samples. We therefore performed the first nationwide seroprevalence study of *Anaplasma* spp., *A. phagocytophilum, Borrelia* spp., *Rickettsia* spp. and *Babesia* spp. in Belgian cattle. We also screened questing ticks for the aforementioned pathogens.

Methods: ELISAs and IFATs were performed on a representative sample set of cattle sera stratified proportionally to the number of cattle herds per province. Questing ticks were collected in areas where the highest prevalence for the forenamed pathogens in cattle serum were observed. Ticks were analyzed by quantitative PCR for *A. phagocytophilum* (n = 783), *B. burgdorferi* sensu lato (n = 783) and *Rickettsia* spp. (n = 715) and by PCR for *Babesia* spp. (n = 358).

Results: The ELISA screening for antibodies to *Anaplasma spp*. and *Borrelia spp*. in cattle sera showed an overall seroprevalence of 15.6% (53/339) and 12.9% (52/402), respectively. The IFAT screening for antibodies against *A. phagocytophilum, Rickettsia* spp. and *Babesia* spp. resulted in an overall seroprevalence of 34.2% (116/339), 31.2% (99/317) and 3.4% (14/412), respectively. At the provincial level, the provinces of Liege and Walloon Brabant harboured the highest seroprevalence of *Anaplasma* spp. (44.4% and 42.7% respectively) and *A. phagocytophilum* (55.6% and 71.4%). East Flanders and Luxembourg exhibited the highest seroprevalence of *Borrelia* spp. (32.4%) and *Rickettsia* spp. (54.8%) respectively. The province of Antwerp showed the highest seroprevalence of *Babesia* spp. (11%). The screening of field-collected ticks resulted in a prevalence of 13.8% for *B. burgdorferi* s.l., with *B. afzelii* and *B. garinii* being the most common genospecies (65.7% and 17.1%, respectively). *Rickettsia* spp. was detected in 7.1% of the tested ticks and the only identified species was *R. helvetica*. A low prevalence was found for *A. phagocytophilum* (0.5%) and no *Babesia* positive tick was detected.

Conclusions: The seroprevalence data in cattle indicate hot spots for tick-borne pathogens in specific provinces and highlights the importance of veterinary surveillance in anticipating the emergence of diseases among humans. The detection of all pathogens, with the exception of *Babesia* spp. in questing ticks, underlines the need of raising awareness among public and professionals on other tick-borne diseases along with lyme borreliosis.

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1. Introduction

Ticks are vectors of a broad range of bacterial, viral and parasitic pathogens that can have a zoonotic potential and are of medical, veterinary and economic importance. Tick-borne diseases are found over most of the world and their incidence is increasing worldwide including in Europe (Klemola et al., 2019). Among these diseases, anaplasmosis, borreliosis, rickettsiosis and babesiosis are noteworthy (Beaujean et al., 2016; De Keukeleire et al., 2018; de la Fuente et al., 2008; Heyman et al., 2010; Semenza and Suk, 2018).

Anaplasma species are gram-negative obligate intracellular bacteria including *A. marginale, A. centrale, A. bovis, A. ovis, A. platys,* and *A. phagocytophilum.* The latter is responsible for human granulocytic anaplasmosis (HGA) and tick-borne fever (TBF) in ruminants (de la Fuente et al., 2007; Dumler et al., 2001; Silaghi et al., 2018; Woldehiwet, 2010). HGA symptoms include fever, headache, myalgias and chills but severe complications associated with opportunistic infections are possible (Dumler et al., 2005, 2001). HGA in Europe including Belgium is suspected to be underdiagnosed given the discrepancy between the seroprevalence and symptomatic rates (Cochez et al., 2011; Dumler et al., 2005, 2001; Scharf et al., 2011). Moderate to high seroprevalence were found in European studies whereas symptomatic infections appear to be rare (Cochez et al., 2011). In cattle, TBF gives rise to symptoms like anorexia, milk drop, respiratory distress, and abortion and hence constitutes a burden for the European farms due to the related significant economic losses (Atif, 2015; Dugat et al., 2015; Lagrée et al., 2018; Woldehiwet, 2006). A new zoonotic *Anaplasma* species, temporarily named *A. capra*, was described in 2015 in humans in China (Li et al., 2015). *A. capra* can also infect ruminants including cattle but the role of these animals in the maintenance of this bacterium is yet to be determined (Koh et al., 2018; Miranda et al., 2021; Peng et al., 2021; Seo et al., 2018).

Lyme borreliosis is the most common tick-borne disease in Europe and is caused by spirochetes of the species complex *Borrelia burgdorferi* sensu lato. This multi-systemic disease can result in humans in skin, neurological and cardiac manifestations if untreated (Chomel, 2015; Stanek et al., 2012; Stanek and Reiter, 2011; Strle and Stanek, 2009). Lyme disease can also occur in cattle and in its acute form it causes symptoms like fever, swollen joints, decreased milk production and abortion (Parker and White, 1992). The prevalence in ruminants is however not known in most European countries including Belgium and little is known on the role of ruminants in the ecology of *Borrelia* (Stefancíkova et al., 2002).

The *Rickettsia* genus comprises species causative of rickettsiosis in humans and animals and is divided in two major groups which are the spotted fever group (SFG) and the typhus group (TG). *Ixodidae* ticks act as vectors, reservoirs or amplifiers of the SFG harbouring at least 30 *Rickettsia* species (Low et al., 2020; Montenegro et al., 2017; Stewart and Stewart, 2021). In Europe, *Rickettsia* species transmitted by ticks include *R. conorii, R. helvetica, R. monacensis, R. slovaca* and *R. raoultii* (Parola et al., 2013; Portillo et al., 2015). SGF infection in humans can result, depending on the species, in mild (such as fever, rash and eschar) to severe and life-threatening clinical signs (Robinson et al., 2019). *Rickettsia slovaca* for instance causes tick-borne lymphadenopathy (TIBOLA) called also *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL) or scalp eschar and neck lymphadenopathy (SENLAT) (Portillo et al., 2015). *Rickettsia slovaca* infection in domestic ruminants has been also previously reported, suggesting a role of these animals in the domestic cycle of this pathogen. Less attention was however given to *Rickettsia* infection in these animals since the infection tends to be mild and self-limiting (Lukovsky-Akhsanov et al., 2016; Ortuño et al., 2012).

Babesiosis is one of the most common infectious diseases of wild and domestic animals worldwide. It is caused by intraerythrocytic protozoa and has been recently considered as possible emerging tick-borne zoonosis (Vannier et al., 2015; Zhou et al., 2014). Human cases in Europe were associated with *B. divergens, B. venatorum* (sp. EU1) and, to a lesser extent, *B. microti*. Human infection is often asymptomatic or mild but severe disease was also reported, mainly in immunocompromised or asplenic individuals (Hussain et al., 2022; Krause, 2019). No clinical cases have been reported so far in Belgium although the three European *Babesia* species were previously detected in the country (Lempereur et al., 2015, 2012, 2011). Cattle and roe deer *(Capreolus capreolus)* are suggested as reservoir hosts for *B. divergens* and *B. venatorum,* respectively (Silaghi et al., 2012). Bovine babesiosis was considered to be the most important tick-transmitted disease in cattle. Although subclinical infections with mild symptoms are quite common, severe cases can also occur and are characterized by symptoms like fever, anemia, hemoglobinuria, anorexia, depression, weakness, cessation of rumination, and an increase in respiratory and heart rate (Purnell and Brocklesby, 1977; Zintl et al., 2003).

In Belgium, country-wide data on the prevalence of *Anaplasma* spp., *A. phagocytophilum, Borrelia* spp., SFG *Rickettsia* and *Babesia* spp. in cattle is mostly missing and little recent information on the prevalence of the forenamed infections in questing ticks is available. This study aimed therefore to assess the seroprevalence of the selected tick-borne pathogens in cattle serum from all over Belgium and their prevalence in questing ticks from targeted areas. This selection was made based on the zoonotic potential of the pathogens and the assumed relevance and the veterinary importance in Europe including Belgium.

2. Materials and methods

2.1. Sampling design and ethics statement

Cattle serum samples used for the estimation of the seroprevalence of *Anaplasma* spp., *Borrelia* spp. *Rickettsia* spp. and *Babesia* spp. were selected from the "Winterscreening campaign 2019-2020". The latter is a yearly serological survey in cattle herds during the winter season for notifiable diseases (such as blue tongue, bovine brucellosis and bovine leucosis). This campaign was of interest for our study since it provided samples from all over Belgium relating to the last vector season. Sample size was calculated based on the expected prevalence known so far in literature (Lempereur et al., 2012; Lernout and Tersago, 2018), together with the sensitivity and the specificity of the applied diagnostic tests as provided by the manufacturer. This allows an estimate of the seroprevalence of the selected pathogens with 95% confidence and a precision of 5% (see Table S1).

In order to obtain a representative country-wide seroprevalence, a randomized sample selection stratified to the number of cattle herds per province (extracted in January 2020 from the Belgian system for animals identification, registration and follow-up "SANITEL") was performed. All Belgian provinces (n = 10) were included in this nation-wide screening. An overview of the number of samples collected from each province can be found in Table S2. No specific ethical dossier had to be filled for this study since the collection of blood from cattle at the farm by a veterinarian is considered as a routine veterinary practice and needs no specific approval from an ethical committee under current European and Belgian legislation (Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes; Belgian Royal Decree of May 2013 relating to the accommodation and care of experimental animals (C 2013/24221, chap I. §4)).

2.2. Serological analyses

The presence of antibodies against *Anaplasma* spp. and *Borrelia* spp. in cattle serum samples was tested using *Anaplasma* antibody test cELISA (VMRD, Pullman, USA) and VetLine Borrelia (NovaTec immunodiagnostica GmbH, Dietzenbach, Germany) ELISA (enzyme-linked immunosorbent assay) kits, respectively, following manufacturer's protocols. Results were only accepted if the internal kit controls fulfilled the prescribed conditions. The *Anaplasma* antibody test cELISA uses

recombinant MSP (rMSP5) to detect antibodies to *A. marginale*, A. *ovis* and A. *centrale* in cattle while a mix of antigens from *B. garinii, B. afzelii* and *B. burgdorferi* sensu stricto is used in the VetLine Borrelia ELISA.

Anaplasma phagocytophilum and *Rickettsia* spp. antibodies were detected in cattle serum samples by IFAT (immunofluorescent antibody tests) using commercial IFA slides (MegaFLUO® *ANAPLASMA phagocytophilum* and MegaFLUO® *RICKETTSIA conorii,* Megacor Diagnostik GmbH, Hoerbranz, Austria) and anti-bovine IgG-conjugates. These commercial IFA slides are coated with *A. phagocytophilum*- and *R. conorii*-infected cells, respectively. Cattle sera were tested in a dilution of 1:40 for *A. phagocytophilum* (Ebani et al., 2008; Lempereur et al., 2012; Bauer et al., 2021) and 1:64 for *Rickettsia* spp. (Eisawi et al., 2017; Lledó et al., 2014). Briefly, 20 µl of diluted serum (in PBS, pH 7.2) and controls were dropped onto the slide on separate antigen wells and incubated in a humid chamber for 30 min at 37°C. The slides were washed with PBS (pH 7.2) for 5 min twice and air dried shortly. Twenty µl of fluorescein-labeled anti-cattle IgG (MegaFLUO® VET, Megacor Diagnostik GmbH, Hoerbranz, Austria) or anti-bovine IgG-FITC (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:300 in PBS containing Evans Blue 0.05% were added to *A. phagocytophilum*- or *R. conorii*-coated slides, respectively. After incubation for 30 min at 37°C, the slides were washed twice with PBS for 5 min, coverslips mounted and examined using a fluorescent microscope (Olympus System Microscope Model BX 40) at 400 × magnification. Negative and positive controls were commercially purchased from Megacor Diagnostik GmbH (Hoerbranz, Austria).

The presence of IgG antibodies against *Coxiella burnetii* in bovine sera was assessed using a commercial ELISA kit (PrioCHECKTM Ruminant Q fever Ab Plate Kit, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, diluted serum samples (at 1:400) were added to wells coated with *C. burnetii* whole-cell antigens and detected with HRP-conjugated G protein (at 1:100 dilution). The optical densities (OD) were read at dual wavelengths of 450-620 nm and samples with S/P% > 40 were considered as positive.

The presence of IgG antibodies against *B. divergens* was tested in cattle sera as previously described by Agoulon et al. (2012). Briefly, sera to be tested (10 µL) were incubated in humid atmosphere for 20 min at 37°C then rinsed twice in PBS before addition of 15 µL anti-bovine IgG-FITC (Sigma-Aldrich, Saint Louis, MO, USA), diluted 1/150 in PBS containing Evans Blue 0.001%, for 30 min at 37°C. Detection of fluorescent parasites corresponding to positive sera was performed using a fluorescence microscope (Leitz Laborlux 8). Each microscopic slide used was concomitantly tested with controls consisting of both a positive and a negative bovine serum diluted at 1:80. Cattle sera were tested at 1:80 and 1:320 dilutions.

2.3. Tick collection

A total of 1,323 questing ticks were collected in June and November 2021 by flagging with a flannel cloth in 11 locations (Fig. 1, Fig. S1). These locations showed to harbour in our seroprevalence study in cattle the highest seroprevalence for the selected tick-borne pathogens. Ticks were then sorted based on collection site, genus, developmental stage and sex and stored at -20°C until homogenization.

2.4. Tick homogenization

Ticks were washed in 70% ethanol for 2 min, rinsed twice in sterile MiliQ water for 2 min each time and dried with filter paper. Individual ticks were then placed in a 2ml Eppendorf tube that contains 450 µl of Dulbecco's modified Eagle's medium (DMEM, GibcoTM Thermo Fisher Scientific, Waltham, MA, USA), 50 µl of chitinase (5mg/ml, Sigmaaldrich®, Saint Louis, MO, USA)) and 2 stainless steel beads (5 mm, Qiagen®, Hilden, Germany). The tubes were afterwards incubated at RT for 30 min and placed in a tissue lyser (TissueLyser II, QIAGEN®, Hilden, Germany) for 5 min at 25Hz. After centrifugation at 10000 rpm, 200 µl of tick homogenate was harvested for DNA extraction.

2.5. DNA extraction and quantitative real-time PCR analyses

DNA from tick homogenates was extracted using the MagMax Total Nucleic Acid Isolation Kit (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA) following manufacturers' protocol. One microliter of the eluted DNA was used to confirm the tick genus by high-resolution melting analysis (HRMA) using a SYBR green based real-time PCRs targeting *5S* and *ITS2* genes (see Table S3). The program of the latter consisted of 1x cycle of 10 min at 95°C followed by 50 cycles of 8 s at 95°C, 5 s at 60°C and 5 s at 72°C. The last step was a melting curve assessment and the results were expressed as melting temperature of the corresponding amplicon (Rousseau et al., 2021b).

DNA samples were further screened for the selected pathogens using previously-reported (Courtney et al., 2004) qPCR assays listed in Table S3. In each run, also negative extraction and negative and positive amplification controls were included. For *A. phagocytophilum* and *B. burgdorferi* s.l., the following amplification program was used: 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 45 s at 60°C. The conditions used for the amplification of *Rickettsia* spp. consisted of 10 min at 95°C followed by 45 cycles of 20 s at 95°C, 20 s at 55°C and 30 s at 72°C. All qPCRs were performed using LightCycler® 480 Probes Master Mix (Roche, Basel, Switzerland) and were carried out on a LightCycler 480 Real-Time PCR system (Roche, Basel, Switzerland). Samples that had a Ct value ≥40 were considered as negative.

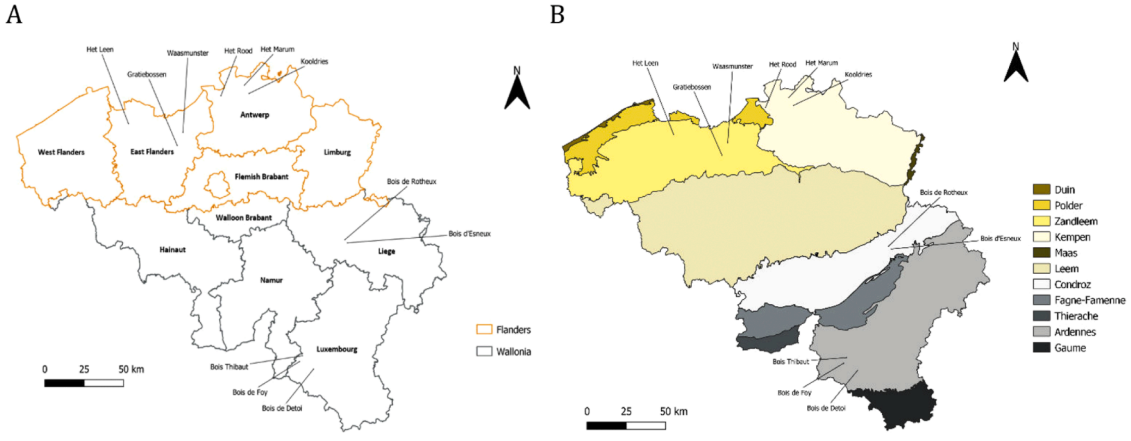

Fig. 1. Locations of the 11 sites where the ticks were collected in 2021 (indicated with arrows in both maps). (A) Indicates the 10 Belgian provinces in bold. (B) Depicts the Belgian ecoregions.

2.6. Conventional PCRs and sequencing

In order to sequence DNA samples found positive in qPCR for *Rickettsia* spp. and identify them to the species level, a 381 bp fragment of the *gltA* gene was amplified by conventional PCR using the Taq DNA polymerase recombinant kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) and previously described primers (Portillo et al., 2017). These are listed in Table S3. The temperature profile used for the PCR reaction was 94°C for 5 min, followed by 35 PCR cycles at 95°C for 20 s, 48°C for 30 s, 60°C for 2 min and final extension at 72°C for 10 min. A conventional PCR targeting the 5S-23S ribosomal RNA intergenic spacer region of *B. burgdorferi* s.l., previously described by Coipan et al. (2013), was used to amplify DNA samples found positive for *B. burgdorferi* by qPCR (see Table S3). All PCR products were then visualized after electrophoretic migration on 1.2% agarose gel and sequencing was outsourced to an external company (Azenta/GENEWIZ, Germany).

Tick DNA samples were screened for *Babesia* spp. by a conventional PCR targeting the 18S rRNA gene of the genus *Babesia* using previously reported primers (Casati et al., 2006). The reaction program consisted of an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s. Amplification was completed by a further 5 min step at 72°C. The ticks screened for *Babesia* spp. were also tested with a PCR targeting the tick 16S rRNA gene using primers described by Baumgarten et al. (1999). This extra step was carried out as an extraction control since *Babesia* pp. screening was performed by a different laboratory (University of Liège).

2.7. Phylogenetic analysis

Obtained sequences were first analyzed using SnapGene Viewer. Forward and reverse reads were then aligned and merged in MEGA 7.0 software to obtain complete sequences. These sequences were aligned to reference sequences reported in GenBank using ClustalW algorithm of MEGA 7.0 software. Sequences were afterwards trimmed and phylogenetic tree was constructed by the Maximum Likelihood method (using Kimura 2-parameter model) implemented in MEGA 7.0. Bootstrap values are based on 1000 repetitions. The tree topology was also confirmed with the Neighbor-Joining (implemented in MEGA 7.0). The new Belgian sequences reported in this study are available in GeneBank under accession numbers OP413851-OP413899. Genetic divergence was computed with MEGA 7.0 using the p-distance model and applying default settings.

2.8. Statistical analysis and maps

The prevalence of the pathogens and the 95% confidence interval (CI) were calculated using the Wilson's method implemented in Epitools website (https://epitools.ausvet.com.au/). Chi square test or Fisher's exact test were used to test the independency between the prevalence of the pathogens in the Belgian regions, the provinces and developmental stages of ticks. Chi square test and Fisher's test were also applied to analyze the prevalence of ticks between seasons. Statistical analyses were performed using GraphPad Prism 9. p-values <0.05 were regarded as significant. Maps showing the sites of tick collection, the Belgian ecoregions, the geographical distribution of the negative and positive cattle samples and the seroprevalence were produced by QGIS®3.4 (Switzerland) using Belgium vector layer in Belgian Lambert 2008 EPSG-projection.

3. Results

3.1. Seroprevalence of the selected tick-borne pathogens in cattle

3.1.1. Anaplasma spp. and A. phagocytophilum antibody detection

Sera of 339 cattle selected from all over Belgium were tested with a cELISA. Here, 53 out of 339 samples tested positive, giving an overall seroprevalence of 15.6% ($CI_{95\%}$:12.2-19.9), with a higher seroprevalence in Wallonia (26.4%, $CI_{95\%}$:19.8-34.3) than in Flanders (8.%, $CI_{95\%}$:5-12.7) (Table 1, Fig. 2). The difference in seroprevalence between regions (Wallonia and Flanders) was statistically significant ($p<0.0001$). At provincial level, samples collected from Liege and Walloon Brabant showed the highest seroprevalence rates of 44.4% ($CI_{95\%}$:29.5-60.4) and 42.7% ($CI_{95\%}$:15.8-74.9), respectively.

The 339 bovine serum samples were also screened for the presence of antibodies against *A. phagocytophilum* using commercial IFA slides to see whether *A. phagocytophilum* was the most important *Anaplasma* spp. present in Belgium and to identify the possible emergence of other *Anaplasma* spp.. This resulted (Table 1, Fig. 2) in an overall seroprevalence of 34.2% ($CI_{95\%}$:29.4 -39.4) with a significant difference between the two regions ($p<0.0001$). A higher seroprevalence was obtained in Wallonia (48.6%, $CI_{95\%}$:40.4-56.8) compared to Flanders (24.1%, $CI_{95\%}$:18.7-30.5). At provincial level, samples from Walloon Brabant had the highest seroprevalence rate of 71.4% ($CI_{95\%}$:35.9-91.8), followed by Liege (55.6%, $CI_{95\%}$:39.6-70.5).

Since serological cross-reactivity between *A. phagocytophilum* and *A. marginale* was previously reported (Dreher et al., 2005), we compared *A. phagocytophilum* antibody detection in cELISA to IFAT. Table 2 shows that 260 out of 339 samples (76.7%) had the same infection status in ELISA and in IFAT (Cohen's Kappa: 0.4). Seventy-one out of 116 positive samples in IFAT were found to be negative in ELISA, leading to a relative sensitivity of 38.8% in ELISA versus IFAT, while 8 samples tested negative in IFAT but positive in ELISA, resulting in a relative specificity of 96.4%. Three out of the 8 samples originated from the province of Liege, 3 were from the Flemish Brabant and 2 from Limburg.

3.1.2. Screening for Borrelia spp. antibodies

A total of 402 serum samples were tested by ELISA to evaluate the seroprevalence of *Borrelia* spp. in Belgian cattle. As shown in Table 1, 12.9% ($CI_{95\%}$:10-16.8) of the tested animals were found positive and a higher prevalence was obtained in Flanders (18.3%, $CI_{95\%}$:13.9-23.7) compared to Wallonia (5.4%, $CI_{95\%}$:2.86-9.9). This difference was statistically significant ($P = 0.0001$). East Flanders exhibited the highest seroprevalence in Belgium (32.4%, $CI_{95\%}$:22.7-43.9). When considering the equivocal samples as positive, the overall seroprevalence of *Borrelia* spp. in Belgian cattle reached 20.9% ($CI_{95\%}$:17.2-25.1, Table S4).

3.1.3. Screening for Rickettsia spp. antibodies

Serum from 317 cattle were screened for anti-*Rickettsia* antibodies using commercial IFA slides. Here, 31.2% ($CI_{95\%}$:26.4-36.5) of the tested animals were positive. Seroprevalence rates of 34.8% ($CI_{95\%}$:27.2-43.3) and 28.6% ($CI_{95\%}$:22.6-35.5) were obtained in Wallonia and Flanders, respectively. Luxembourg exhibited the highest seroprevalence (54.8%, $CI_{95\%}$:37.8-70.8), with statistically significant differences towards Liege ($p = 0.01$), Namur ($p = 0.01$), Limburg ($p = 0.001$) and Antwerp ($p = 0.02$) provinces (Table 1, Fig. 2).

3.1.4. Serological cross-reactions

We investigated whether the lack of agreement between IFAT and ELISA in assessing the exposure to *A. phagocytophilum* (Table 2) could be explained by the possible serological cross-reactivity between *Rickettsia* and *Anaplasma* (belonging to the same order of Rickettsiales). It turned out that 45.4% the samples that were positive for *Anaplasma* antibodies in IFAT but negative in cELISA were also positive for *Rickettsia* antibodies by IFAT. Since serological cross-reaction between *C. burnetii* (the causative agent of Q fever) and *Anaplasma* was previously described (Graham et al., 2000), we further subjected the samples presenting divergent *Anaplasma* infection status between ELISA and IFAT to an ELISA that detects antibodies against Q fever. However, only 12.7% of the divergent samples were also positive for Q fever (data not showed).

Table 1
Overview of the seroprevalence of the selected tick-borne pathogens in Belgian cattle.

	Screening of *Anaplasma* spp. by ELISA		Screening of *A. phagocytophilum* by IFAT		Screening of *Borrelia* spp. by ELISA		Screening of *Rickettsia* spp. by IFAT	
Provinces	Total	Seroprevalence (%)	Total	Seroprevalence (%)	Total	Seroprevalence (%)	Total	Seroprevalence (%)
Antwerp	36	11.1 ($CI_{95\%}$:4.4-25.3)	36	41.7 ($CI_{95\%}$:27.1-57.8)	42	11.9 ($CI_{95\%}$:5.2-25)	33	24.2 ($CI_{95\%}$:12.8-41.0)
Limburg	22	18.1 ($CI_{95\%}$:7.3-38.5)	22	36.4 ($CI_{95\%}$:19.7-57.0)	26	7.7 ($CI_{95\%}$:2.1-24.1)	21	9.5 ($CI_{95\%}$:2.6-28.9)
East Flanders	60	8.3 ($CI_{95\%}$:3.6-18.1)	60	20 ($CI_{95\%}$:11.8-31.8)	71	32.4 ($CI_{95\%}$:22.7-43.9)	56	33.9 ($CI_{95\%}$:22.9-47)
West Flanders	61	0	61	19.7 ($CI_{95\%}$:11.6-31.3)	72	16.7 ($CI_{95\%}$:9.8-26.9)	57	33.3 ($CI_{95\%}$:22.5-46.3)
Flemish Brabant	20	15 ($CI_{95\%}$:5.2-36)	20	5 ($CI_{95\%}$:0.9-23.6)	24	4.2 ($CI_{95\%}$:0.7-20.2)	18	27.8 ($CI_{95\%}$:12.5-50.9)
Flanders	**199**	**8 ($CI_{95\%}$:5-12.7)**	**199**	**24.1 ($CI_{95\%}$:18.7-30.5)**	**235**	**18.3 ($CI_{95\%}$:13.9-23.7)**	**185**	**28.6 ($CI_{95\%}$:22.6-35.5)**
Walloon Brabant	7	42.9 ($CI_{95\%}$:15.8-74.9)	7	71.4 ($CI_{95\%}$:35.9-91.8)	8	0	6	33.3 ($CI_{95\%}$:9.7-70)
Hainaut	40	30 ($CI_{95\%}$:18.1-45.4)	40	55 ($CI_{95\%}$:39.8-69.3)	48	4.2 ($CI_{95\%}$:1.1-14)	38	39.5 ($CI_{95\%}$:25.6-55.3)
Namur	25	8 ($CI_{95\%}$:2.2-25)	25	32 ($CI_{95\%}$:17.2-51.6)	29	6.9 ($CI_{95\%}$:1.9-22)	23	17.4 ($CI_{95\%}$:7-37.1)
Liege	36	44.4 ($CI_{95\%}$:29.5-60.4)	36	55.6 ($CI_{95\%}$:39.6-70.5)	43	7 ($CI_{95\%}$:2.4-18.6)	34	23.5 ($CI_{95\%}$:12.4-40)
Luxembourg	32	12.5 ($CI_{95\%}$:5-28.1)	32	40.6 ($CI_{95\%}$:25.5-57.7)	39	5.1 ($CI_{95\%}$:1.4-16.9)	31	54.8 ($CI_{95\%}$:37.8-70.8)
Wallonia	**140**	**26.4 ($CI_{95\%}$:19.8-34.3)**	**140**	**48.6($CI_{95\%}$:40.4-56.8)**	**167**	**5.4 ($CI_{95\%}$:2.9-9.9)**	**132**	**34.8 ($CI_{95\%}$:27.2-43.3)**
Belgium	**339**	**15.6 ($CI_{95\%}$:12.1-19.9)**	**339**	**34.2 ($CI_{95\%}$:29.4-39.4)**	**402**	**12.9 ($CI_{95\%}$:10-16.6)**	**317**	**31.2 ($CI_{95\%}$:26.4-36.5)**

The bold values highlight the data of the 2 regions in Belgium, each composed of several provinces

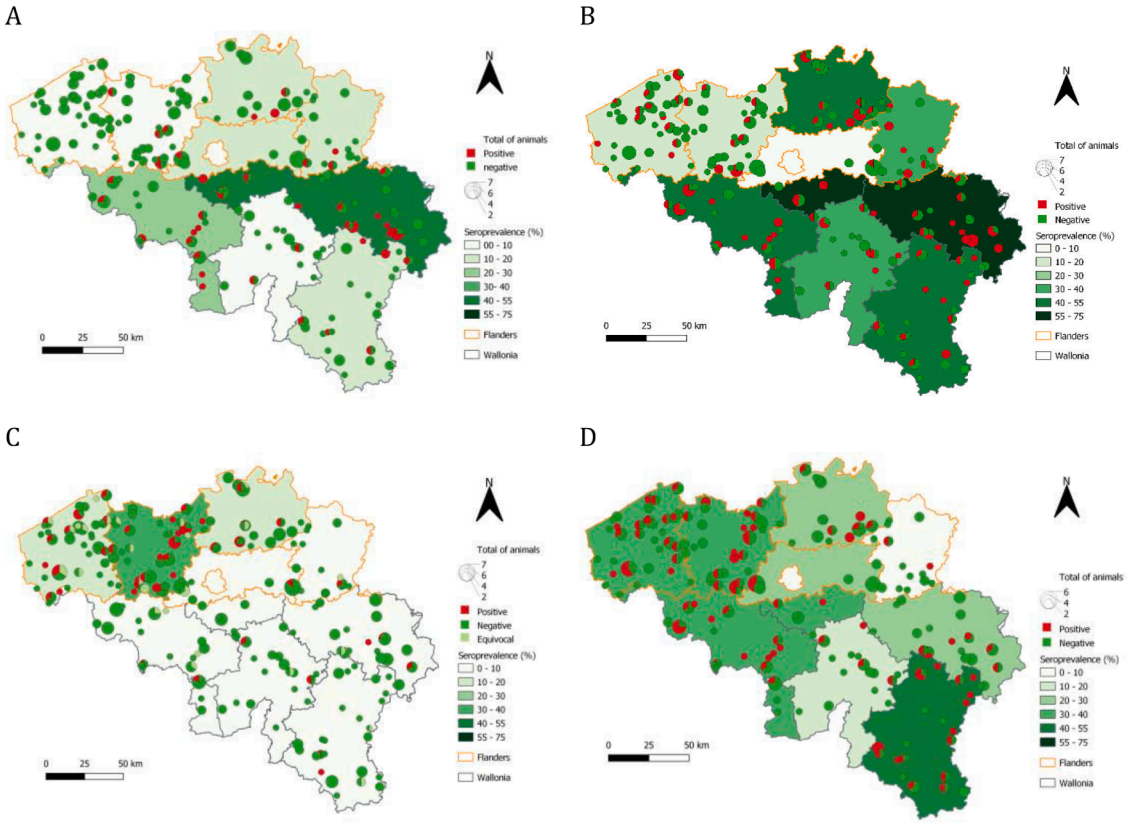

Fig. 2. Geographical distribution of positive and negative animals for antibodies to Anaplasma spp. (A), A. phagocytophilum (B), Borrelia spp. (C) and Rickettsia spp. (D) among the Belgian provinces. The pie chart depicts the number of positive and negative animals per farm and its size proportional to the total of animals from each farm.

Table 2
Relative sensitivity and specificity of *Anaplasma* antibody detection in ELISA versus IFAT

		Infection status of *Anaplasma* spp. in ELISA				
		Pos	Neg	Total	Relative sensitivity	Relative specificity
Infection status of A. *phagocytophilum* in IFAT	Pos	45	71	116	**38.8%**	**96.4%**
	Neg	8	215	223		
	Total	53	286	**339**		

3.1.5. Screening for Babesia divergens antibodies using IFAT

The IFAT screening of bovine sera for the presence of anti-*B. divergens* IgG resulted in 14 positive samples, indicating an overall seroprevalence of 3.4% ($CI_{95\%}$:2.03-5.62) for Belgium. A seroprevalence of 3.7% (9 positive samples out of 242) was found in Flanders, and 5 out of 170 samples (3%) were found positive in Wallonia. The highest seroprevalence was observed in Antwerp (11%, 5 positive samples out of 44), in Liège (7%, 3 positive samples out of 44), in Luxembourg (5%, 2 positive samples out of 39), and then in Flemish Brabant and East Flanders (both 4%, with 1 positive sample out of 24 and 3 positive samples out of 73, respectively). In the provinces of Limburg, West Flanders, Walloon Brabant, Hainaut and Namur none of the samples tested positive (Fig. 3).

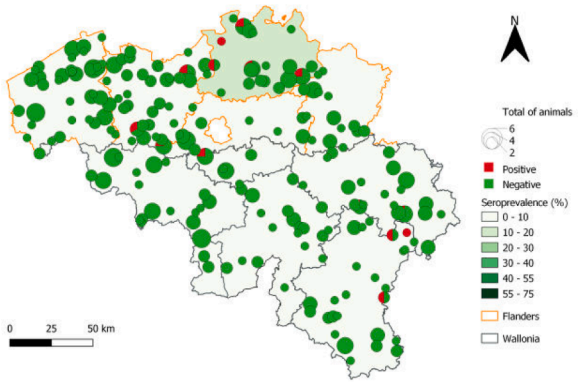

Fig. 3. Geographical distribution of positive and negative samples for antibodies against *Babesia divergens*. The pie chart depicts the number of positive and negative animals per farm and its size proportional to the total of animals from each farm.

3.2. Prevalence of the selected tick-borne pathogens in ticks

Before screening the 1,323 sampled ticks for the selected pathogens, their genus was confirmed by high resolution melting analysis (HRMA) using a SYBR green based real-time PCRs. This resulted in 99.92% ticks of the genus *Ixodes* and 0.08% ticks of the genus *Dermacentor*.

3.2.1. A. phagocytophilum

A total of 783 *Ixodes* ticks were tested for the presence of *A. phagocytophilum* by a qPCR targeting the *msp2* gene: 386 questing ticks collected from 2 sites in the province of Liege, where the seroprevalence of *Anaplasma* in cattle showed to be high (Table 1), and 397 ticks from 3 sites in the province of East Flanders (see Fig. S1). The latter presented a lower seroprevalence of *Anaplasma* in cattle (Table 1). The qPCR results in Table 3 show that only 4 out of 783 ticks tested positive for *A. phagocytophilum* resulting in an overall prevalence of 0.5% ($CI_{95\%}$:0.2-1.3). The 4 positive ticks included 1 female from Bois de Rotheux, 2 nymphs from Bois d'Esneux and 1 nymph from Gratiebossen.

3.2.2. Borrelia burgdorferi s.l

The overall infection rate of *B. burgdorferi* s.l. (Table 3) in the 783 ticks (Fig. S1) tested by the *23S rRNA*-targeting qPCR was 13.4% ($CI_{95\%}$:11.2-16). Unlike the results of the seroprevalence of *Borrelia* in cattle, where samples from the province of East Flanders showed a significantly higher seroprevalence (Fisher's Exact test, p=0.0023) compared to the province of Liège (Table 1), a prevalence of 13.7% was obtained for the ticks from the province of Liege and a prevalence of 13.1% was found in the ticks from East Flanders (Table 3). The prevalence rates of *B. burgdorferi* s.l. within the life stages of ticks were 14.7% and 12.8% in adult ticks and nymphs, respectively.

The seasonal variation in the prevalence of *B. burgdorferi* s.l. was analyzed for the ticks from the sites in the province of East Flanders. Based on Table S5, *B. burgdorferi* s.l. prevalence in the ticks collected in summer was 15.7% while a prevalence of 9.3% was found in the autumnal ticks. The variation of *B. burgdorferi* s.l. infection between summer and autumn was not analyzed for the ticks from the province of Liege given the low number (n=14) of ticks collected in this province in autumn.

A total of 35 sequences of the 5S-23S gene were obtained allowing hence the identification of 4 *Borrelia* genospecies. *Borrelia afzelii* was the most dominant genospecies (65.71%), followed by *B. garinii* (17.14%), *B. burgdorferi* (s.s.) (11.43%) and *B. valaisiana* (5.71%).

3.2.3. Rickettsia spp

A qPCR targeting the *gltA* gene was used to test for the presence of *Rickettsia* spp. in a total of 715 ticks. This included 318 ticks collected from 3 sites in the province of Luxembourg, given its high prevalence of *Rickettsia* in cattle sera (Table 1), and 397 ticks collected from 3 sites in the province of East Flanders (Fig. S1). *Rickettsia* DNA was detected in 7.1% ($CI_{95\%}$:11.2-16) of the tested ticks (Table 4).

DNA samples of 51 *Rickettsia*-positive *Ixodes* ticks were sequenced and a total of 49 sequences of *gltA* gene was successfully obtained and analyzed. The phylogenetic tree (Fig. 4) shows that 48 out of 49 Belgian sequences were identified as *R. helvetica*. These sequences exhibited at the nucleotide level 99.6-100% identity to the reference sequence (GenBank: U59723) and were 99.9% identical to each other. One Belgian sequence (1132-BE) was not identified and when performing a nucleotide blast it shared 82.7% identity with "*Candidatus* Rickettsiella viridis" (GenBank: AP018005). The robustness of our analysis was evaluated by alternatively performing the phylogeny using the Neighbor-Joining method. This resulted in the same classification of all sequences (data not shown).

Table 4
Prevalence of *Rickettsia* spp. in ticks from the provinces of Luxembourg and East Flanders.

Province	Ticks	Total	Pos	Neg	Prevalence (%)
Luxembourg[c]	Nymph	98	7	91	7.1
	Male	99	9	90	9.1
	Female	121	9	112	7.4
	Total	**318**	**25**	**293**	**7.7**
East Flanders[b]	Nymph	242	13	229	5.4
	Male	84	9	75	10.7
	Female	71	4	67	5.6
	Total	**397**	**26**	**371**	**6.5**
	Overall	**715**	**51**	**664**	**7.1**

[c] 3 sites: Bois Thibau (49°55′00.7"N 5°07′08.0"E), Bois de Detoi (49°49′55.4"N 5°12′52.8"E) and Bois de Foy (49°52′46.3"N 5°05′27.3"E)
[b] 3 sites: Het Leen (51°09′56.2"N 3°33′47.7"E), Gratiebossen (51°03′17.4"N 4°00′14.0"E) and Waasmunster (51°06′20.2"N 4°00′41.1"E)

Table 3
Prevalence of *A. phagocytophilum* and *B. burgdorferi* s.l. in ticks from the sites in the provinces of Liege and East Flanders.

		A. phagocytophilum			*B. burgdorferi* s.l.		
Province	Ticks	Total	Pos	Prevalence (%)	Total	Pos	Prevalence (%)
Liege[a]	Nymph	303	2	0.6	303	43	14.2
	Male	47	0	0	47	4	8.5
	Female	36	1	2.8	36	6	16.7
	Total	**386**	**3**	**0.8**	**386**	**53**	**13.7**
East Flanders[b]	Nymph	242	1	0.4	242	27	11.2
	Male	84	0	0	84	16	19
	Female	71	0	0	71	9	12.7
	Total	**397**	**1**	**0.2**	**397**	**52**	**13.1**
	Overall	**783**	**4**	**0.5**	**783**	**105**	**13.4**

[a] 2 sites: Bois de Rotheux (50°32′13.1"N 5°30′29.8"E) and Bois d'Esneux (50°30′42.8"N 5°32′05.3"E)
[b] 3 sites: Het Leen (51°09′56.2"N 3°33′47.7"E), Gratiebossen (51°03′17.4"N 4°00′14.0"E) and Waasmunster (51°06′20.2"N 4°00′41.1"E)

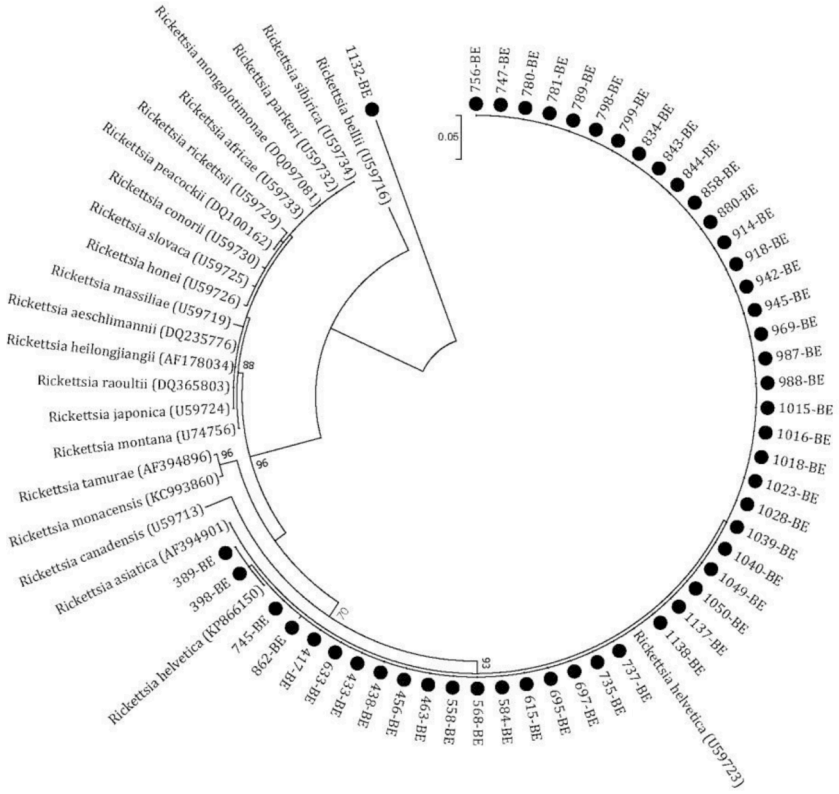

Fig. 4. Phylogenetic tree inferred using the partial *gltA* gene (222 nt) of 49 Belgian *Rickettsia* sequences and 22 reference sequences. Phylogenetic analysis was carried out using the Maximum Likelihood method with the Kimura 2-parameter model. The bootstrap values were obtained from 1000 replicates and only values ≥70% are shown. The Belgian isolates sequenced in this study are marked with a black circle.

3.2.4. Babesia spp

A total of 358 ticks were tested for the presence of *Babesia* spp. This comprised 222 ticks collected at sites within the province of Antwerp, where the seroprevalence in cattle was shown to be the highest (Fig. 3), and 136 ticks collected at sites in the province of East Flanders (Fig. S1). No *Babesia* positive tick was however detected.

4. Discussion

There is a paucity of information on the prevalence of *Anaplasma* spp. (including A. *phagocytophilum*), *Borrelia* spp., *Rickettsia* spp. and *Babesia* spp. in cattle in Belgium. Our study was to our knowledge the first to evaluate the seroprevalence of these pathogens in cattle from Belgium. In this study, antibodies to *Anaplasma* spp. were detected by ELISA in 15.6% of the Belgian cattle. This is in line with the seroprevalence of 16.5% in cattle from Central North Morocco (Hamou et al., 2012). Although this ELISA is a test recognized by the OIE, there is a lack of *Anaplasma* seroprevalence surveys with this ELISA in Europe which makes it difficult to compare to neighboring countries. Cattle sera were also screened by IFAT for *A. phagocytophilum* antibodies and a seroprevalence of 34.2% was obtained. This is higher compared to other publications from Europe (Ebani et al., 2008) but consistent with previously reported data from Belgium. Lempereur et al. (2012) estimated by IFAT (using also 1:40 as cut-off dilution) the seroprevalence of *A. phagocytophilum* in cattle from 8 farms in Southern Belgium (Wallonia) to be at 30-77%, depending on the season. Tavernier et al. (2015) also found a high *A. phagocytophilum* seroprevalence (46.2%) in roe deer from Flanders (the Northern part of Belgium).

Although 260 out of 339 samples (76.7%) obtained the same infection status, divergent results between *A. phagocytophilum* antibody detection in ELISA and in IFAT were found. Seventy-one animals were found positive in IFAT but negative in ELISA, whereas eight samples were positive in ELISA but negative in IFAT. The commercial IFA slides are coated with *A. phagocytophilum* infected cells and detect preferentially antibodies against *A. phagocytophilum*. The ELISA is a competitive cELISA based on a monoclonal antibody that recognizes major surface protein 5 (MSP5) from *A. marginale, A. ovis* and *A. centrale*. This protein is conserved within the genus *Anaplasma* and serological cross-reactivity with *A. phagocytophilum* was previously reported when using this cELISA (Dreher et al., 2005). However, another study reported that serum samples from humans or dogs infected with *A. phagocytophilum* did not cross-react with rMsp5 of *A. marginale* when tested with the cELISA but reacted to rMsp5 of *A. marginale* in an indirect-ELISA (Strik et al., 2007). This was explained by a steric hindrance of the monoclonal antibody by other antibodies directed against epitopes adjacent to the one recognized by the monoclonal antibody (Strik et al., 2007). Another explanation to the low sensitivity of the cELISA compared to the IFAT could be that cattle were exposed to infections with other rickettsial pathogens that may induce antibodies cross-reactive with *A. phagocytophilum* proteins, leading to false-positive IFAT results (Al-Adhami et al., 2011). This possibility is supported by the fact that 45.4% of the samples that were positive for *Anaplasma* in IFAT but negative in ELISA were also found seropositive for *Rickettsia* spp. by IFAT. Additionally, 12.7% of these divergent samples were also positive for antibodies against *C. burnetii*. Furthermore, non-specific fluorescence seems to be common in IFA assay and is attributable to antibodies adhering to infected erythrocytes (Aubry and Geale, 2011). In the absence of a specific "gold standard" serological method for the diagnosis of anaplasmosis, epidemiological data would gain from combining results from IFAT and cELISA to identify positive reactors to *A. phagocytophilum*. While cELISA would miss some cases, the data with IFAT should be interpreted taking into account the serological intra-species cross-reactivity and the cross-reaction with other Rickettsiales (Bauer et al., 2021; Dreher et al., 2005).

The fact that eight cattle samples (originating from three provinces) were found positive in *Anaplasma* spp. ELISA and negative in *A. phagocytophilum* IFAT may suggest the emergence in Belgium of new *Anaplasma* spp. such as *A. capra*. The latter was reported in cattle in South Korea and Malaysia, in sheep and goats in China, but also in red deer (*Cervus elaphus*), swamp deer (*Rucervus duvaucelii*), sheep and goats in France (Jouglin et al., 2022, 2019; Koh et al., 2018; Peng et al., 2018; Seo et al., 2018; Yang et al., 2017). However, further analyses (beyond the scope of this study) are needed to support this suggestion since no information on the serological cross-reactivity of this specie is available.

The results of both ELISA and IFAT showed a significantly higher *Anaplasma* seroprevalence in Wallonia compared to Flanders. This difference might be related to several factors including differences between both regions in the vegetation and habitat suitability for ticks and the density of wildlife reservoir hosts. Rousseau et al. (2021a) highlighted the importance of forests, fragmented landscapes and wild hosts as environmental determinants of *A. phagocytophilum* infection in cattle. A vegetation hospitable to ticks and their hosts would likely contribute to a higher abundance of ticks and thus a higher probability of infection. *Ixodes ricinus* is the main vector of *A. phagocytophilum* and the most common tick species in Belgium. Woodlands and forests constitute its main habitat and according to Tack et al (2012) *I. ricinus* is more abundant in oak forests than in pine forests. Forests cover only about 11% of the total area in Flanders while in Wallonia the forest cover is over 30% (Vandekerkhove, 2013). Moreover, unlike Flanders, where 30% of the forests are pine plantations and only 8% are oak forests, 18% of the Walloon forests are oak forests and only 3% are pine plantations (Vandekerkhove, 2013). This suggests that *I. ricinus* might be more abundant in Wallonia than in Flanders, although remains purely speculative and worthy further investigation. Red deer and roe deer play an important role in maintaining large populations of *I. ricinus* but also as important reservoir hosts for *A. phagocytophilum* (Hamšíková et al., 2019; Ruiz-Fons and Gilbert, 2010). The prevalence of *A. phagocytophilum* was reported as positively linked to the density of red deer (Mysterud et al., 2018, 2013). The latter often present high percentages of seropositivity for *A. phagocytophilum* (46.2%) and in Europe they are currently suspected to carry strains infecting cattle (Ebani et al., 2008; Lagrée et al., 2018). In Belgium, the density of red deer is different between both regions, probably due to the different forest cover mentioned above. Red deer populations are established in Wallonia and live mainly in the large forested areas of the provinces of Liege and Luxembourg (Prévot and Licoppe, 2013). In Flanders, individual red deer can occasionally be observed but only in Voeren (province of Limburg). Altogether these differences could likely explain the higher prevalence of *A. phagocytophilum* in Wallonia compared to Flanders.

The province of Liège showed to harbour the highest seroprevalence rates of *Anaplasma* spp. (44.4%) and *A. phagocytophilum* (55.6%). This is in agreement with the data reported by Rousseau et al. (2021a) and not surprising since this area seems to be endemic for this pathogen. The first case of bovine *A. phagocytophilum* infection in Wallonia was detected in 2005 in cattle from the province of Liège (Guyot et al., 2011) and subsequent studies also supported the presence of seropositive cattle originating from this province (Delooz, 2014; Lempereur et al., 2012). The province of Liège's territory is composed mainly of the Condroz, the Fagnes-Famenne and the Ardennes ecoregions (Fig. 1) characterized by fragmented landscapes and a high proportion of forests and grasslands. These seem to be important environmental determinants of *A. phagocytophilum* infection in cattle (Rousseau et al., 2021a).

An overall seroprevalence of 12.9% was obtained for *Borrelia* spp. in cattle. A comparison of this result with the European trend is difficult as comparable studies are rare. Stefancíkova et al., 2002 estimated the seroprevalence of *B. burgdorferi* s.l. in dairy cattle from Slovakia at 25.2%, which is higher compared to our result. Unlike *Anaplasma*, we found a significantly higher *Borrelia* spp. seroprevalence in cattle from Flanders compared to Wallonia. This seems consistent with the hypothesis of the dilution effect predicting a reduced prevalence of *B. burgdorferi* in areas with high density of red deer (Mysterud et al., 2013; Rosef et al., 2014). Deer, including red deer, are considered to be as non-competent reservoir for *B. burgdorferi,* thanks to their immune system (Kurtenbach et al., 2002). Their presence at high density may have an effect on diverting ticks from feeding on competent reservoir hosts (like rodents) hence decreasing potential infection transmission and *Borrelia* prevalence (Richter and Matuschka, 2006; Sala and De Faveri, 2016).

Rickettsia spp. antibodies were sought in cattle sera by IFAT and a prevalence of 31.2% was obtained in this study. Little information on rickettsial infection in animals is available, especially in cattle. Antibodies against SFG *Rickettsia* were detected in cattle in Japan at a prevalence of 9.6% (Jilintai et al., 2008). In Spain, the seroprevalence of *R. slovaca* in bullfighting cattle was 65% (Ortuño et al., 2012) and in Sudan SFG *Rickettsia* seroprevalence in cattle was 64.4% (Eisawi et al., 2017). Cattle serve as a host for adult *I. ricinus* and transovarial transmission of *Rickettsia* spp. has been demonstrated (Hauck et al., 2020; Sprong et al., 2009). These high seroprevalence including ours suggest that the role of these animals, often in close contact to humans, in maintaining rickettsial natural life cycles needs further investigation. Luxembourg showed to be the province with the highest seroprevalence (54.8%). This suggests that the latter may harbour the most suitable environment for the bacterium, its vector and reservoir hosts. This province lays mainly within the Ardennes and the Gaume ecoregions (Fig. 1) and is the most forested area. Alone it accounts for 43% of the total forest area of Wallonia. Based on Hermy et al. (2021), the number of tick bites reported per 100,000 inhabitants between 2016 and 2021 was the highest in this province which may suggest a highest density of ticks in this area. Ticks are vectors but also the main reservoir for most SFG *Rickettsia* (Tomassone et al., 2018). This altogether may be a contributor factor to the high *Rickettsia* seroprevalence in this province.

Within this study, the overall IFAT-determined seroprevalence of *Babesia* spp. in cattle was 3.4%. This is lower than previously reported data in Belgium (Lempereur et al., 2012). The highest seroprevalence was found in the province of Antwerp (11%). This result may at first sound surprising given the records of *B. divergens* in the Southern part of Belgium (Wallonia). However, it may also be indicative of the emergence of this pathogen in this area particularly since ticks from the province of Antwerp were previously found positives for *B. microti* and *B. venatorum* (Lempereur et al., 2012, 2011).

The prevalence of the selected tick-borne pathogens in questing ticks was also addressed in this study using ticks collected in areas that showed the highest seroprevalence rates in cattle. This resulted in a prevalence of 0.5% for *A. phagocytophilum* in ticks. This is in agreement with the rather low number of confirmed HGA cases in Belgium. Similar low prevalence rates were reported in a meta-analysis in questing ticks from Ukraine (0.4%), China (0.6%), Austria (0.7%), Netherlands (0.8%), Poland (0.9%) and Germany (1%) (Karshima et al., 2022). The prevalence of *A. phagocytophilum* in ticks removed from humans and originating from Belgium was also low (1.8%)(Lernout et al., 2019). The low prevalence found in questing ticks is in contrast to the high seroprevalence obtained in cattle. The mechanical transmission of *Anaplasma* spp. through biting flies can also occur (Atif, 2015) and might explain this discrepancy. No related data are however available for Belgium to corroborate this statement. The high seroprevalence in cattle could also be associated to antibodies persistence after past infections. Based on Bakken et al. (2002), *A. phagocytophilum* antibodies can last for more than three years in some infected humans but no similar follow-up was reported for cattle.

In line with the overall mean prevalence in Europe (13.7%) (Rizzoli et al., 2011), 13.4% of the ticks tested in the present study were positive for *B. burgdorferi* s.l.. The prevalence of *B. burgdorferi* s.l. in Belgium was previously estimated at 23% in questing ticks from the province of Namur (Misonne et al., 1998) and at 12% in questing ticks from the province of Hainaut (Kesteman et al., 2010). Other studies in the country reported a prevalence of 10.2% in ticks fed on cats and dogs (Claerebout

et al., 2013) and of 14% in ticks removed from humans (Lernout et al., 2019).

When analyzing the variation of *B. burgdorferi* s.l. prevalence in ticks between seasons, an infection rate of 15.7% was obtained in the ticks from summer (June) while only 9.3% of the tested autumnal ticks (October) were positive. This corresponds to the dynamics of human tick bites reported in Belgium between 2016 and 2021, exhibiting a peak in June (Hermy et al., 2021). The age of the tick and the survival of the pathogens within the tick over season may affect the infection rate. It was suggested that when tick moulting and activity take place in the warmest period of the year, the survival of the bacteria was negatively affected since the immune system of the tick would be more effective in this period. This would henceforth result in a lower prevalence in autumn (Mysterud et al., 2013).

In parallel with other studies in Belgium and neighboring countries (Kesteman et al., 2010; Lernout et al., 2019; Quessada et al., 2003; Waindok et al., 2017; Rousseau et al., 2022), *B. afzelii* and *B. garinii* were the most identified genospecies in this study (65.7% and 17.1%, respectively). We also recorded two other genospecies namely *B. burgdorferi* s.s., and *B. valaisiana* (at 11.4%, and 5.7%, respectively). These genospecies were previously reported in Belgium but at different proportions (Kesteman et al., 2010; Lernout et al., 2019; Rousseau et al., 2022). This difference may be related to temporal and spatial variations between the studies or diversity in reservoir hosts. The four genospecies found are generally related to different competent hosts and different clinical manifestations. *Borrelia afzelii* is rodent-associated and is linked mostly to skin manifestations (Herzberger et al., 2007; Stanek and Reiter, 2011; Van Duijvendijk et al., 2015). *Borrelia garinii* and *B. valaisiana* rely mainly on birds as reservoir hosts. The former is mostly associated with neuroborreliosis while the pathogenicity of the latter is not clear. *Borrelia burgdorferi* s.s. is a host generalist and appears to be the most arthritogenic (Van Duijvendijk et al., 2015).

The prevalence of *Rickettsia* spp. in ticks was estimated at 7.1% in the present study with *R. helvetica* being the detected specie. This is in agreement with Lernout et al. (2019) who found a prevalence of 6.8% for *R. helvetica* in ticks removed from humans from all over the country. Nevertheless, previous studies in Belgium reported higher *R. helvetica* prevalence in questing ticks (16.9%), in ticks fed on cats and dogs (14%) and on songbirds (22%) (Claerebout et al., 2013; Heylen et al., 2017, 2016). In 2015 and 2016, respectively 24 and 25 human cases of rickettsiosis were diagnosed in Belgium but no confirmed autochthonous infections have been reported yet (Geebelen et al., 2022; Rebolledo et al., 2017). Although evidence for disease causality is still missing, *R. helvetica* should not be overlooked and further prospective studies need to be performed to gain more insight into its possible pathogenicity (Azagi et al., 2020).

No *Babesia* spp. positive tick was found in our study. Although no human clinical cases of babesiosis have been reported so far in Belgium, this result was not expected since *Babesia* spp. was previously detected in the country. Its prevalence was previously estimated at 1.5% in ticks fed on humans and at 7.9% in questing ticks from areas with known babesiosis. Moreover, it represented 1.3% in ticks fed on cats and dogs and 14.6% in cattle-derived ticks (Claerebout et al., 2013; Lempereur et al., 2012; Lernout et al., 2019). Taken together, these results indicate that attention should be paid to the possibility of future cases of bovine babesiosis in Belgium, despite an apparently rather low circulation rate of *Babesia* spp. among cattle and ticks at present.

5. Conclusion

This study was the first to screen for the presence of antibodies to *Anaplasma* spp. (including A. *phagocytophilum*), *Borrelia* spp., *Rickettsia* spp and *Babesia* spp. in cattle at a country-wide scale in Belgium. It also provided an up-to-date information on the prevalence of these pathogens in questing ticks from targeted areas. The high seroprevalence rates of *Anaplasma* spp., *A. phagocytophilum, Borrelia* spp. and *Rickettsia* spp. in specific provinces indicate hot spots for these tick-borne pathogens and underlines the need to closely follow up their epidemiological status. Studying the patterns of exposure of cattle to tick-borne pathogens could help anticipating and preventing the emergence of these diseases among humans. Except for *Babesia* spp., all pathogens were detected in field-collected ticks, albeit to a different extent. A follow up of prevalence variations over time is recommended and there is a need to increase awareness among the public, veterinarian and healthcare professionals on the presence in Belgium of other tick-borne diseases along with Lyme borreliosis.

CRediT authorship contribution statement

Nadjah Radia Adjadj: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Mickaël Cargnel:** Methodology, Writing – review & editing. **Stefaan Ribbens:** Resources, Writing – review & editing. **Christian Quinet:** Resources, Writing – review & editing. **Laurence Malandrin:** Methodology, Writing – review & editing. **Bernard Mignon:** Methodology, Writing – review & editing. **Marcella Mori:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

Authors' contributions

All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102146.

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