University of Veterinary Medicine
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Prevalence of *Coxiella burnetii* in dairy cattle and farm workers and associated bovine reproductive disorders in the Central European region

Ph.D. thesis

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Abbreviations

AI	Artificial insemination
APSW complex	Abortion, premature delivery, stillbirth and weak offspring
BVD	Bovine Viral Diarrhea
BoHV-1	Bovine Herpes Virus 1
CDS	Coding DNA sequence
CFT	Complement fixation test
CI	Confidence interval
Ct	Cycle threshold
CRO	Croatia
CZ	Czech Republic
DNA	deoxyribonucleic acid
IFA	Indirect immunofluorescence assay
IHC	Immunohistochemistry
IgG	Immunoglobulin G
ELISA	Enzyme-linked immunosorbent assay
EPL	Early pregnancy loss
HU	Hungary
LCV	large-cell variant
LPSs	Lipopolysaccharides
Mbp	Megabase pair
MST	Multispacer sequence typing
OR	Odds ratio
PCR	Polymerase chain reaction
PPL	Percentage pregnancy loss
PSPB	Pregnancy-specific protein B
SCV	small-cell variant
SK	Slovakia
SLO	Slovenia
SRB	Serbia
ST	sequence type
TMR	Total mixed ration
qPCR	Real-time polymerase chain reaction

1. Summary

Q fever is a zoonotic disease present worldwide, which was first described in 1937 in slaughterhouse workers in Australia. As the cause of the illness was unknown, workers showing flu-like symptoms were diagnosed with 'Query' fever, this is where the name of the disease comes from. The causative agent of the disease is *Coxiella burnetii*, an obligate intracellular Gram-negative bacterium. Knowledge about *C. burnetii* and associated diseases has expanded intensely since its first description, revealing a wide host spectrum (domestic animals, reptiles, ticks, birds, and marine mammals) and several ways of shedding the bacteria (by birth products, urine, feces and milk). Infected animals generally remain asymptomatic, but various reproductive disorders such as infertility, premature birth, stillbirth, abortion or pregnancy loss can also be associated with the pathogen. *C. burnetii* has a high zoonotic potential, and human infections were often connected with outbreaks in domestic ruminants. Therefore, monitoring domestic ruminants for *C. burnetii* infection is important, as cattle, sheep and goats are the main reservoir of the disease and the main sources of human infections. Serological methods (e.g., complement fixation test, enzyme-linked immunosorbent assays (ELISA) detecting Phase II (acute infection) and Phase I (chronic infection) antibodies), molecular biological (e.g., real-time polymerase chain reactions (PCR, qPCR, nested-PCR) and conventional microbiological methods are in use for the detection and diagnosis of *C. burnetii* infections. Based on the comparison of multiple diagnostic methods, the combined use of serological and molecular biological tools (preferably ELISA and PCR assays) was suggested for the reliable diagnosis of Q fever.

Current information on Q fever in dairy cattle farms in the Central and Eastern European region was limited. Therefore, the aim of the present study was to assess the prevalence of *C. burnetii* in dairy cattle herds of different sizes in six countries from this area, by examining bulk tank milk samples with ELISA and real-time PCR tests. We found that *C. burnetii* seroprevalence varies among the countries. In Croatia the level of seropostivity of the investigated herds was 100.00%, the Czech Republic 98.55%, Hungary 97.61%, Serbia 70.83%, Slovakia 90.56%, and Slovenia showing 62.50%. *C. burnetii* specific ELISA showed 100.00% positivity in all examined countries in herds of 250 or more milking cows. Based on our results we can conclude that the growing number of farms managing a large number of animals, where cattle density is high, correlates with the increasing prevalence of *C. burnetii* in the region.

The prevalence of Q fever is highly variable by country. The main reservoirs of the disease are the same domestic ruminant species everywhere, but the epidemiological profile depends on the features of the specific reservoir. Our large-scale study demonstrated the importance of Q fever in different host species. *C. burnetii* seroprevalence rates varied among the animal species tested in Hungary. Seropositivity by ELISA was 47.2% in cows and 25.5% in small

ruminants, with 23.5% in sheep and 31.0% in goats. Antibodies to *C. burnetii* were not detected in the tested zoo animals. This study has demonstrated that Q fever is spread most widely in dairy cattle in Hungary but sheep and goats also appear to pose a major risk for humans.

It has been demonstrated that IgG Phase I and Phase II antibodies to *C. burnetii* are higher in Hungarian dairy farm workers than those described in several international seroepidemiological studies among different occupational groups in other European countries. Veterinarians are the most exposed to infection, but inseminators and animal caretakers are at a similarly high risk of infection in industrial dairy farms. The high *Coxiella* burden in dairy farms underlines the importance of controlling the disease. It has also been demonstrated that high *C. burnetii* seroprevalence among dairy farm workers correlated with a high prevalence of *C. burnetii* in Hungarian dairy herds.

Several studies reported that infertility, premature birth, stillbirth, abortion or pregnancy loss can also be associated with this pathogen. We found a higher seropositivity rate (80.5%) in cows that had lost their pregnancy. Moreover, seropositivity rate was even higher (94.4%) in the first-bred cows that had lost their pregnancy at an early stage. ELISA-positive pregnant and aborted cows were further investigated by the complement fixation test (CFT). The average individual seropositivity in dairy herds as detected by CFT (Phase II) was 66.6% in previously ELISA-positive animals that had lost their pregnancy and 64.5% in pregnant animals. Phase I seropositivity rate (50.0%) was higher in cows with pregnancy loss compared to pregnant animals (38.5%). The high prevalence of *C. burnetii* in dairy farms might potentially contribute to an increased risk of pregnancy loss.

It was also an aim of the current thesis to compare of the occurrence of *C. burnetii* in retained fetal membranes and normally separated placentas. A further objective was to identify the *C. burnetii* multispacer sequence typing (MST) genotypes occurring in Hungary and Slovakia. The results of the thesis indicate that prevalence and DNA load of *C. burnetii* in retained fetal membranes is significantly higher than in normally separeted placentas, and it may act as a possible risk factor for human infections mostly in workers and veterinarians who come into contact with retained placentas. Five out of the ten samples from retained placentas showing the strongest positivity (Ct 11.92–18.28) were genotyped by MST based on ten loci. This revealed sequence type (ST) 61, which had not been found previously in Hungary and Slovakia. The new ST61 and the ST20 genotype previously found in Hungary are still the primary causes of bovine coxiellosis in the region. We conclude that the high *C. burnetii* DNA load found in retained fetal membranes in Central European dairy farms may be not only an important risk factor for human infection but may also be associated with the retention of fetal membranes.

Összefoglalás

A Q-láz világszerte előforduló zoonózis, amelyet 1937-ben írtak le először Ausztráliában, vágóhídi munkások között. Az ismeretlen oktanú, magas lázzal, influenza szerű tünetekkel járó, járványos megbetegedések kórokozójaként a *Coxiella burnetii,* Gram-negatív intracelluláris baktériumot azonosították. Ismereteink a kórokozóról nagymértékben bővültek a betegség első leírása óta, mind a gazdaspektrum, mind a betegség terjedésének tekintetében. Számos emlősfaj mellett a baktériumot kimutatták már többek között hüllőkben és tengeri emlősökben is. A kórokozó számos módon ürülhet a gazdaszervezetből, többek között vizelettel, bélsárral, tejjel és magzatburokkal is. A fertőzött állatok gyakran tünetmentesek, azonban számos szaporodásbiológiai problémát, mint vetélés, magzatburok retenció, méhgyulladás, korai magzatvesztés is összefüggésbe hozták a kórokozó jelenlétével. Az emberi Q-lázas megbetegedések elsődleges forrásai azonban a házi kérődző állományok. A kórokozó három legfontosabb rezervoárja a kecske, a juh és a szarvasmarha, így a humán megbetegedések fő forrásai is ezek a fajok, ennél fogva a *C. burnetii* fertőzöttségük mértékének folyamatos monitorozása is elengedhetetlen. A Q-láz fertőzés kimutatása különféle szerológiai próbák segítségével történhet (indirekt immunfluoreszcenciás vizsgálattal, komplementkötési próbával és ELISA segítségével, mely Fázis I-es és II-es antigének ellen termelt ellenanyagok kimutatására szolgál), valamint molekuláris biológiai vizsgálatokkal (valós idejű polimeráz láncreakció) és hagyományos mikrobiológiai módszerekkel (baktérium izolálás 3-as biztonsági fokozatú laboratóriumi körülmények között). Célszerű több diagnosztikai módszert együttesen használni a még pontosabb diagnózis felállítása érdekében.

A közép-kelet-európai régió tejelő szarvasmarha telepeinek Q-láz fertőzöttségének mértékéről hiányosak az ismereteink. Vizsgálatunk célja így a *C. burnetii* prevalenciájának meghatározása volt a régió hat országából, különböző méretű tejelő szarvasmarha telepekről származó tanktej mintákból ELISA és PCR vizsgálatok segítségével. A különböző országokban különböző prevalenciát találtunk (Horvátország 100%, Csehország 98,55%, Magyarország 97,61%, Szerbia 70,83%, Szlovákia 90,56% és Szlovénia 62,5%). A *C. burnetii* specifikus ELISA vizsgálatok pedig minden vizsgált országban 100%-os pozitivitást mutattak, ahol az állományban a tejelő állatok létszáma 250 vagy annál több volt. Vizsgálataink alapján megállapítható, hogy azokon a tejelő szarvasmarha telepeken, ahol nagy mennyiségű állatot tartanak és az állatsűrűség nagyobb, a *C. burnetii* prevalenciája is magasabb.

A Q-láz előfordulásának gyakorisága eltérő a különböző országokban. A fő gazdafajok ugyanazok az állatfajok mindenhol, de a betegség elterjedtségének mértékét a rezervár fajok sajátosságai befolyásolják. Ennek vizsgálatára nagy populációra kiterjedő és több gazdafajt is érintő vizsgálatot végeztünk Magyarország összes régiójában. A különböző kérődző

gazdafajokban eltérő *C. burnetii* szeroprevalenciát találtunk ELISA vizsgálatokkal. Szarvasmarhák esetében 47,2%-os míg a kiskérődzők esetében 25,5%-os (23,5% juhok és 31% kecske) szeroprevalenciát találtunk felmérő vizsgálataink során. Az állatkerti kérődzők esetében a kórokozóval szembeni ellenanyagok nem voltak kimutathatóak. Kutatásainkkal igazoltuk, hogy Magyarországon a Q-láz kórokozója a szarvasmarha állományokban a legszélesebb körben elterjedt a kérődző fajok között, de a juh és kecske állományok is közegészségügyi kockázatot jelenthetnek.

Jelenlegi kutatásunkkal igazoltuk azt is, hogy a tejelő szarvasmarha telepen dolgozók esetében *C. burnetii* átfertőzöttség mértéke jelentősen magasabb a különböző foglalkozási csoportokban, összehasonlítva a különböző országok hasonló kutatásaival. Vizsgálataink során beigazolódott, hogy a szarvasmarha praxisban dolgozó állatorvosok a leginkább veszélyeztetett csoport a *C. burnetii* fertőzöttség szemponjából, de az inszeminátorok és állatgondozók is különösen kitettek a kórokozóval történő fertőződésnek. A tejelő szarvasmarha telepek magas *Coxiella* terheltsége miatt különösen fontos a betegség kontrollálása, mivel jelenlegi kutatásunk igazolta, hogy a tejelő telepek magas *Coxiella* szeroprevalenciája nemcsak az állatoknál, hanem az ott dolgozóknál is hasonlóan magas.

Számos tanulmány szerint kapcsolat áll fenn a kórokozó jelenléte és a szarvasmarhák szaporodásbiológiai problémái között, úgymint infertilitás, koraellés, vetélés, korai magzatvesztés. Kutatásunkban magasabb szeropozitivást találtunk (80,5%) azoknál az állatoknál, akik elvesztették magzatukat a vemhesség korai stádiumában, mint akik vemhesek maradtak. Az első termékenyítés esetén még magasabb volt a szeropozitivátás aránya (94,4%). Az ELISA pozitív állatokat komplementkötési (KK) módszerrel tovább vizsgáltuk és magasabb szeropozitivitási arányt találtunk Fázis I antigén ellen termelt ellenanyagok tekintetében a magzatot vesztett állatok tekintetében (50%), mint a vemhesen maradt állatoknál (38,5%). Ezek alapján megállapítható, hogy a *C. burnetii* jelenléte a tejelő szarvasmarha telepeken feltételezhetően emeli a magzatvesztés kockázatát.

Kutatásunk további célja volt, hogy összehasonlítsuk a kórokozó előfordulásának gyakoriságát a magzatburok retenciós és normál módon eltávozott placentákban. További célunk volt, hogy meghatározzuk magyarországi és szlovákiai szarvasmarha magzatburkokból származó mintákból a kórokozó genotípusát. A kutatás eredményeképpen megállapítható, hogy a retenciós placentákban szignifikánsan magasabb volt a kórokozó előfordulása, így nagyobb kockázatot jelent azon dolgozók számára a kórokozóval való fertőződés, akik ezekkel a retenciós placentákkal dolgoznak. A legerősebb pozitivitást mutató mintákból (Ct 11,92–18,28) végeztünk MST genotipizálást. Az öt cotyledon minta MST vizsgálata során egy új (ST61) szekvencia (ST) előfordulását mutattuk ki, amely eddig sem Magyarországon sem pedig Szlovákiában nem fordultak elő. Az új szekvencia (ST 61) és az előző kutatásokban talált ST20 genotípusú kórokozók melyek a szarvasmarhák coxiellózisában szerepet játszanak a régióban. Feltételezhető, hogy a kórokozó gyakori előfordulása a retenciós placentákban nemcsak a humán fertőzöttség szemponjából fontos, hanem szerepe lehet a magzatburok retenció kialakulásában is.

2. Introduction

2.1 History

C. burnetii, the agent of “query fever” or Q fever is an important zoonotic pathogen, which was first described in Australia in 1935 by Edward Holbrook Derrick, an Australian pathologist. He investigated an outbreak of a febrile illness that occurred in abattoir workers in Brisbane, Australia (Derrick, 1937). Two Australian scientists Frank Macfarlane Burnet (Figure 1) and Mavis Freeman isolated an intracellular organism in 1937 from specimens received from Derrick that were passaged in mice (Burnet and Freeman, 1983). It was originally identified as a species of Rickettsia and the organism then was named *Rickettsia burnetii.* At the same time an American bacteriologist Herald Rea Cox studying the ecology of Rocky Mountain spotted febrile disease in western Montana, isolated an agent from a tick that he characterized as a rickettsia, just like Burnet in Australia. Because the agent of Q fever was markedly different from other Rickettsiae, Philip proposed a new genus, Coxiella. *C. burnetii* is the only member of the genus (Philip, 1948).

Figure 1. Portrait of Sir Frank Macfarlane Burnet (1899-1985) on an Australian stamp (https://touchstamps.com)

2.2 Aetiology

C. burnetii is an obligate intracellular, pleomorphic Gram-negative small coccobacillus (0.2 to 0.4 µm wide and 0.4 to 1 µm long) that causes Q fever. The bacterium is not stained by Gram stain and is generally stained using the Gimenez method in clinical specimens (Eldin at al., 2017). The genome size of *C. burnetii* is around 2.0 Mbp. Genome analysis revealed many genes with different potential roles in adhesion, invasion and intracellular trafficking (Seshadri at al., 2003). This obligate intracellular bacterium encodes an unusually high number of basic

proteins, which are possibly responsible for face osmotic and oxidative stress condition and buffer of the acidic environment of the phagolysosome (D'Amato et al., 2016). *C. burnetii* strains contains four large, autonomously replicating plasmids (QpH1, QpRS, QpDV, or QpDG) and a QpRS-like chromosomally integrated sequence of unknown function. These plasmids have a possible role in the virulence of *C. burnetii* (Shengdong et al., 2020). *C. burnetii* genome exhibites 83 pseudogenes, they are characterized by the presence of 29 insertion sequence (IS) elements. In the bacteria the IS elements are dispersed around the chromosome but not found on the plasmid (Seshadri et al., 2003). Thus the insertion sequence *IS1111* present in the genome of *C. burnetii* is routinely used for confirmation of Q fever cases. The organism genome is predicted to encode 2,134 CDSs, 719 (33.7%) of which are hypothetical (Eldin et al., 2017).

The agent has two cell variants corresponding to a biphasic developmental cycle. The large-cell variant (LCV) (size: >0.5 µm), is the form in which it replicates and is sensitive to environmental stress. The small-cell variant (SCV) (size: 0,2 to 0,5 µm) is characterised by high environmental stability and can remain infectious in the extracellular environment for more than a year in highly resistant spore-like forms (McCaul and Williams, 1981; Howe and Mallavia, 2000; Schimmer et al., 2012).

2.3 Genomic aspects of *C. burnetii*

Pathogenicity and virulence of *C. burnetii* depends on the infected animal species, the route of infection, the *C. burnetii* strain, and the inoculum size (Eldin et al., 2017). Genomic aspects of the bacteria play an important role in determining the virulence of the agent. The intracellular nature of *C. burnetii* made the search for virulence determinants very difficult. Genomics, and more particularly comparative genomics studies, have demonstrated that the word "Q fever" covers a large range of epidemiological and pathogenicity characteristics, depending mainly upon the genetic characteristics of the *C. burnetii* strain involved. Eight genomic groups (I-VIII) have been described by genome comparison studies. They grouped *C. burnetii* isolates according to their genetic composition (Dragan et al., 2020). The Dugway strains exhibited the largest genome (2,158,758 bp chromosome and 54,179 bp QpDG plasmid). These strains were isolated from rodents in Dugway, Utah, USA, in the 1950s. The strains reside in a distinct genomic group of *C. burnetii* and were considered avirulent despite having the largest genomes of the Coxiella genus. Phylogenetically, the Dugway strains appear to represent a more primitive genomic group which did not go through the genome reduction associated with pathogenic *C. burnetii* strains (Beare et al., 2017). Cb175 from Cayenne, French Guiana, had the smallest genome, due to an unique 6,105-bp deletion in the coding region for the type 1

secretion system (T1SS) (1,989,565 bp chromosome, 37,398 bp QpH1 plasmid). Cb175 and other strains of the mutispacer sequence type (MST) 17 specific for French Guiana are the most virulent strains ever described. They cause the highest prevalence of community-acquired pneumonia in the world. Consequently, the observed genome reduction is probably a mechanism leading to increased virulence in this *C. burnetii* clone (Eldin et al., 2017). The MST genotyping of a high number of strains from different geographical areas helps us better understand the epidemiology of *C. burnetii* from one region to another and identify epidemic clones. Currently, some MST are spread across the five continents, while others are very specific to one geographical area like MST 17 in French Guiana (Santos et al., 2012; Tilburg et al., 2012; D'Amato et al., 2016). Geographical distribution of *C. burnetii* detected genotypes are visualized in Figure 2.

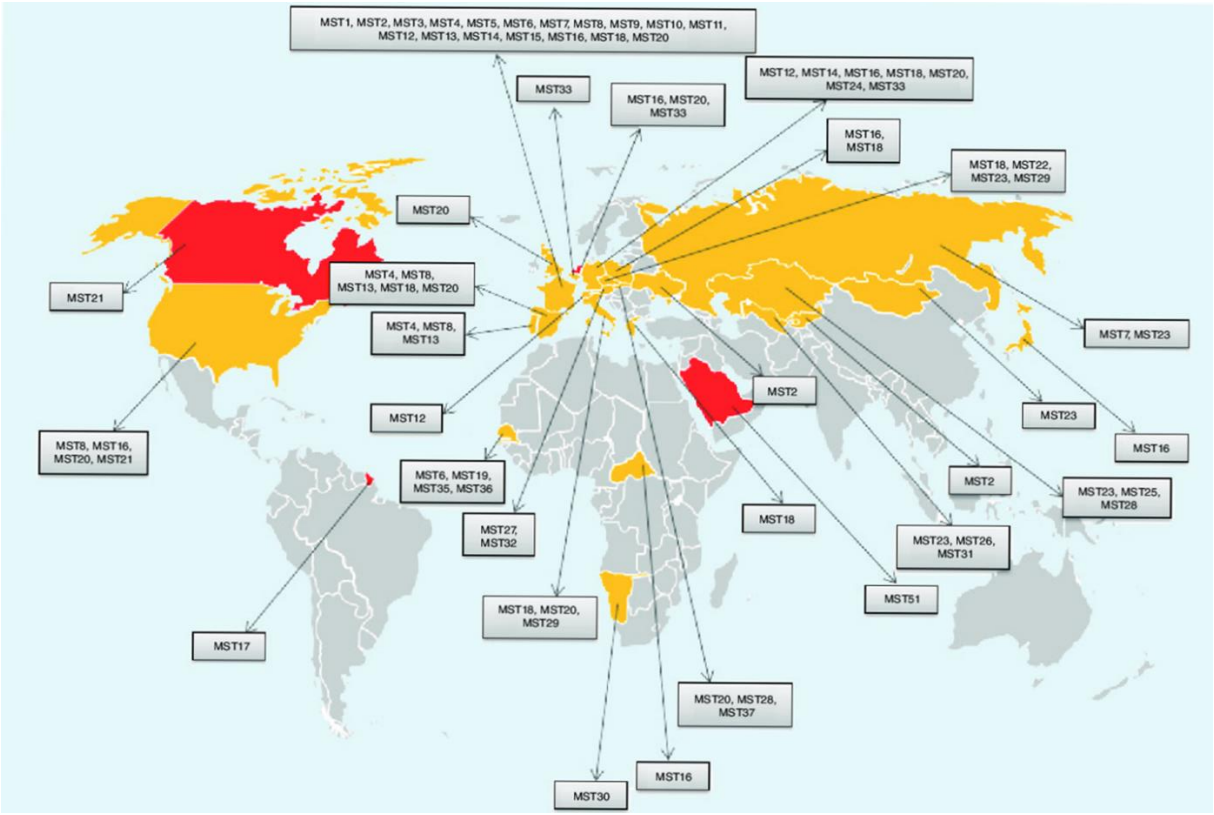

Figure 2. Geographical distribution of *C. burnetii* detected genotypes. In red, countries where only one clone is circulating. In yellow, countries where other MST genotypes have been described (D'Amato et al., 2016).

2.4 Geographic distribution of Q fever in Central and Eastern Europe

The first diagnosis and report of Q fever in Hungary in cattle and sheep took place in 1956 (Romváry et al., 1957). Two large outbreaks were recorded in dairy cattle farms accompanied by several human cases in 1977 (EPINFO, 2014). Rády et al., published that abortion caused by *C. burnetii* occurred sporadically in cattle stocks while large numbers of abortions took place within a short time in some sheep flocks in Hungary (Rády et al., 1987). The latest major outbreak, registered in 2013, originated from a sheep flock in Southern Hungary, where 70 laboratory-confirmed human cases were reported (Gyuranecz et al., 2014). Some *C. burnetii* abortion cases were reported in cattle and sheep but only a single caprine abortion case was diagnosed and reported until now in Hungary (Rády et al., 1985; Szeredi et al., 2006).

In Croatia, the infection is considered to be endemic with 20 to 70 human cases reported yearly, and outbreaks among humans connected mostly with the presence of the pathogen in small ruminants (Cvetnic et al., 2005; Medic et al., 2005). Accordingly, *C. burnetii* was detected (by PCR) at higher numbers in a genotyping study from aborted small ruminants (sheep, n=48/681; goats, n=218/739) than from aborted cattle (n=44/1604) in this country (Racic et al., 2014)

In the Czech Republic the first case of Q fever was reported in 1953 (Patocka and Kubelka, 1953). In dairy farms seroprevalence was determined to be in the range of 4-19% by complement fixation test in the sera of newly dried-off cows originating from 14 herds in Northern Moravia, Czech Republic (Literak and Kroupa, 1998).

In Serbia, first reports of Q fever were published in the 1950s (Jovanovic et al., 1950), and the pathogen is considered to be endemic in the region (Medic et al., 2012; Debeljak et al., 2018). A recent epidemiological study detected low seroprevalence in cattle (8.3%) (Debeljak et al., 2018).

Q fever was first reported in Slovakia in 1954, and then sporadic cases were observed in the following decades (Serbezov et al., 1999). Sheep farms were assumed to be the source of infection in major outbreaks among humans and an increasing seroprevalence of *C. burnetii* was observed in sheep in the country (Dorko et al., 2008; Dorko et al., 2010).

In Slovenia, the first Q fever outbreak in humans was reported in 1954, and the latest, most important outbreak was detected in 2007 in veterinary students and teachers, associated with a training course on a sheep farm (Grilc et al., 2007). Most recently, the prevalence of the pathogen has been determined in the country by examining questing and feeding ticks and blood samples of sheep and cattle. The seroprevalence of *C. burnetii* in cattle based on ELISA tests was 48%, while the DNA of the pathogen was detected in 8% (*n*=4/50) of the animals' blood samples (Knap et al., 2019).

2.5 Epidemiology

Q fever is a zoonotic disease and is distributed worldwide except for New Zealand. Knowledge on *C. burnetii* and associated diseases has expanded intensely since its first description, revealing a wide host spectrum (domestic animals, reptiles, ticks, birds and marine mammals) and several ways of shedding the bacteria (Eldin et al., 2017). The main reservoirs of the disease are the same domestic ruminant species everywhere, but the epidemiological profile depends on the features of the specific reservoir. Even though the agent has a broad reservoir range including many domestic and wild mammals, the main reservoirs are cattle, sheep, and goats (Maurin and Rault, 1999). Many seroepidemiological studies proved the role of these three species, and some authors have also reported *C. burnetii* infection in zoo and wild animals (Enright et al., 1971; Kruse et al., 2004; Clemente et al., 2008). Cattle, sheep, and goats are the main sources of human infections: *C. burnetii* is mainly shed by infected domestic ruminants via birth products, vaginal secretions, faeces, and milk (Eldin et al., 2017). *C. burnetii* contaminated dust particles may also remain infectious for long periods after shedding (Joulié et al., 2015). Q fever outbreaks in humans have been generally associated with small ruminants (Tissot-Dupont et al., 1999; Van den Brom et al., 2013), but there are several reports of sporadic human disease cases closely linked to cattle. Strains of *C. burnetii* (strain RSA 493 / Nine Mile I) were isolated from a guinea pig on which field collected Rocky Mountain *Dermacentor andersoni* ticks had fed, suggesting that transmission through tick bites may also occur (Duron et al., 2015a). However, some novel *Coxiella*-like organisms were described in non-vertebrate species particularly in ticks and it is suggested that the common ancestor of *C. burnetii* originated from *Coxiella* hosted by soft ticks. Some tick species were found to harbour maternally-inherited *Coxiella*-like organisms engaged in symbiotic interactions, but their relationships to the Q fever pathogen remain unclear (Duron et al., 2015b). Some cases were reported about possible transmission from pigeons which spread *C. burnetii* infected ectoparasites to humans and it was suspected that bacteria were transmitted by tick bites, but there is no solid evidence of arthropod-borne transmission of the disease to humans (Stein and Raoult, 1999; Maurin and Rault, 1999).

It is still not clear if consumption of dairy products from *C. burnetii*-infected animals can lead to foodborne Q fever in humans, although some studies reported the disase after consumption of raw milk (Eldin et al., 2017).

The most important and primary mode of human infection is the inhalation of infected aerosols of *C. burnetii.* Bacterial infection may occur after contamination with infected animals' birth products, abortion materials, hides, wool, manure, etc., mainly at the time of parturition (Maurin and Rault, 1999). *C. burnetii* is shed by ruminants by faeces which contaminates the bedding material, thus contaminated manure may be another source of human Q fever (Hermans et al., 2014). Bacterial aerosols can be spread for at least 30 km by the wind, thus, *C. burnetii* infections may occur in humans without any evident contact with animals (Tissot-Dupont et al., 2004).

2.6 Pathogenesis

One of the most important characteristics of *C. burnetii* is the phase variation which is an antigenic variation of lipopolysaccharides (LPSs). *C. burnetii* isolated from acutely infected animals, or humans is present in a wild virulent form, with a smooth full length LPS with O antigen sugars, named Phase I. After several passages in embryonated hen eggs or cell cultures, the bacterium shifts from the highly infectious Phase I to an non infectious Phase II form which expresses truncated rough LPS (Angelakis and Rault, 2010). Microscopically the two forms are indistinguishable, but the serological response is different. The immune response to the Phase II antigen is much more significant during the acute infection compared with the chronic infection, where titres to the Phase I antigen are higher (Oyston and Davies, 2011).

Alveolar macrophages are the target cells of *C. burnetii* following aerosol transmission, bacteria passively enter these cells by actin-dependent phagocytosis. *C. burnetii* is characterized as a stealth pathogen that enters cells without alerting the immune system (van Schaik et al., 2013).

The virulent forms of *C. burnetii* survive inside the human monocytes, whereas the avirulent forms are eliminated. The adaptation of *C. burnetii* to intracellular life is closly linked with the acidic pH of its phagosome. Both forms are found in phagosomes (van Schaik et al., 2013).

C. burnetii is internalized and multiplies within eukaryotic cells in phagosomes, which fuse rapidly with lysosomes to form phagolysosomes. The early phagolysosomes fuse progressively to form a large unique vacuole (Angelakis and Rault, 2010).

Primary multiplication of the bacteria takes place in the regional lymph nodes, an ensuing bacteraemia lasts for 5–7 days and the organism then localizes in the mammary glands and the placenta of pregnant animals (Babudieri, 1959). In pregnant goats and other ruminants, the trophoblast cells of the allantochorion are also primary target cells for *C. burnetii* (Brom and van den Engelen, 2015). Haematogenous spread results in the bacteria infecting the liver,

spleen, bone marrow, and other organs, causing granulomatous lesions (Woldehiwet, 2004). *C. burnetii* persists in fixed macrophages and its intracellular survival is due to the subversion of some macrophage functions and the impairment of T-cell responses (Mege et al., 1997). As the protective T-cell mediated immunity is depressed, humoral immunity plays a central role in the elimination of this intracellular organisms from the infected animals (Woldehiwet, 2004). In goats after the inoculation, *C. burnetii* Phase II specific antibodies, both IgM and IgG, can be detected after two weeks and remain increased for up to 13 weeks post-infection. Antibodies directed against *C. burnetii* Phase I increase as well, but about four weeks later than Phase II specific antibodies (Roest et al., 2013). In humans, serological follow-up until four years after acute Q fever diagnosis showed that Phase I IgG antibody titres decreased slightly and Phase II antibody titres remained high among possible chronic Q fever patients. It is still unclear which factors cause the persistence of high Phase I antibody titres in those patients. After the biggest Q fever outbreak in the Netherlands a large scale study demonstrated that possible chronic Q fever patients have high Phase II IgG levels as well as measurable IgG Phase I antibody titres, even after 48 months of follow-up (Jajou et al., 2014; Wielders et al., 2015).

C. burnetii has several routes of shedding. Bacteria are mainly shed by birth products, birth fluids and placenta (Guatteo and al., 2006). *C. burnetii* may also be shed by ruminants via vaginal mucus, milk, faeces, urine and semen. The placenta of infected animals contains the highest concentration of bacteria, and it is the most important source of human infection (Guatteo et al., 2007). In milk, sporadic and persistent shedding were the most frequent kinetic patterns among dairy cattle reported by Guatteo et al. (2006). Goats excreted the bacteria mainly in milk (Rodolakis et al., 2007).

2.7 Clinical signs and Pathology

APSW complex (abortion, premature delivery, stillbirth and weak offspring) is a well-known manifestation of Q fever in cattle (Agerholm et al., 2013). However, these dramatic clinical manifestations -mainly abortion- are predominantly seen in sheep and goats. Cattle are frequently asymptomatic although clinically infected cows develop infertility, metritis and subclinical mastitis (Barlow and Rauch, 2008; Porter et al., 2011; De Biase et al., 2018). *C. burnetii* was found to be significantly associated with placentitis but with mostly mild changes in the cotyledons (Bildfell et al., 2000; Hansen et al., 2011). Placental necrosis and fetal bronchopneumonia were also significantly associated with the presence of *C. burnetii* in the trophoblasts (Bildfell et al., 2000).

Nowadays, there is an increased awareness of Q fever as an economically important disease on industrial dairy cattle farms. Infected animals usually remain asymptomatic, but the

presence of the bacteria may lead to economic losses through reduced fertility (To et al.,1998.; Vourvidis et al., 2021).

Several human Q fever outbreaks are related to small ruminants worldwide (Eldin et al., 2017). Coxiellosis in small ruminants is generally asymptomatic but goats and sheep are the species in which abortions, stillbirths, and early neonatal mortality have most frequently been documented. Generally abortion occurs in late pregnancy. In The Netherlands there was a 75-fold increase in the goat population between 1985 and 2009, and the country faced one of the largest Q fever outbreaks in the World (Eldin et al., 2017). Several well-documented Q fever abortions were diagnosed in dairy goat and sheep farms during this period. Abortions were mostly seen without signs of general illness, but some goats were temporarily a little sluggish and had reduced appetite. Some goats developed mild endometritis after abortion. Several kids were weak, with low body weight and high mortality after normal parturition, and some new-born animals suffered from respiratory and digestive tract disorders. Treatment of pregnant goats with oxytetracyclines did not reduce the abortion rate (Roest et al., 2012). In *C. burnetii* infected placentas dark-red colouration and necrosis of cotyledons or intercotyledonary areas are observed. Surface was sometimes covered by a greyish-white or reddish-brown secretion (Szeredi et al., 2006) (Figure 2). Although several human Q fever outbreaks are related to sheep, abortions are not always observed (Brom et al., 2015).

Figure 3. Placenta from a case of caprine abortion induced by *C. burnetii*. Note the congested cotyledons with dark-red colouration and the oedematous, reddish intercotyledonary areas (L. Szeredi)

2.8 Human Q fever

Acute Q fever is usually characterized by flu-like symptoms however, the main characteristic of the disease is its clinical polymorphism. The acute clinical manifestation is influenced by the primary infection which causes a wide variety of clinical symptoms (Eldin et al., 2017). The incubation period lasts about 2 to 3 weeks. According to most studies the major clinical manifestation of acute Q fever is a febrile illness, which is associated with severe headaches, myalgias, arthralgias and cough (Tissot-Dupont and Raoult, 2007). Main symptoms of the acute disease are fever, pulmonary signs of varying severity and elevated liver enzyme levels, which can occur concurrently. Prolonged fever is usually observed and is accompanied by severe headaches (Angelakis and Rault, 2010). Atypical pneumonia is one of the most commonly recognized forms of acute Q fever and is mostly clinically asymptomatic or mild. Pneumonia is the major manifestation of acute disease in many countries (Canada, Spain, Switzerland) while hepatitis is the predominant form of Q fever in some endemic regions such as France, Isreal or Taiwan (Eldin at al., 2017).

Acute pericarditis and acute myocarditis are rare forms of the primary infection. Meningitis and meningoencephalitis have been also reported in some cases but these symptoms are also rare, just like bone marrow involvement or acute lymphadenitis (Eldin at al., 2017).

Chronic Q fever can develop from a primary acute infection in about 1% to 5% of patients (Ghaoui et al., 2019). It may develop several months to many years after initial infection (Tulassay, 2010). Typically, the heart is the most commonly involved organ, followed by the vascular system. Q fever derived endocarditis is the most frequently reported form of persistent chronic *C. burnetii* infection (Angelakis and Rault, 2010). The clinical presentation of *C. burnetii* endocarditis is nonspecific, and patients can present symptoms such as isolated relapsing fever, chills, night sweats, weight loss, and hepatosplenomegaly (Tulassay, 2010). The aortic and mitral valves are mostly involved and Q fever prosthetic valve endocarditis is also reported in many cases (Eldin et al., 2017). Positive *C. burnetii* PCR in blood or tissue or IFA titer of 1:1,024 for *C. burnetii* Phase I IgG, and definite endocarditis raises the suspicion for chronic form of the disaese. The prevalence of chronic Q fever is probably underestimated in most developing countries, where microbiological tools for diagnosis are lacking (Ghaoui et al., 2019).

2.9 Diagnosis

Clinical signs and clinical manifestation of Q fever are often subclinical in both humans and animals, thus the use of different kinds of laboratory methods are key points in diagnosing and monitoring Q fever. The methods avaialable for the diagnosis of Q fever and their purpose according to OIE are summerised in Table 1 (OIE, 2018).

Table 1. Test methods avaialable for the diagnosis of Q fever and their purpose according to OIE (OIE, 2018).

Method	Purpose					
	Population: freedom from infection	Individual animal: freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
PCR	+++	n/a	+++	+++	++	+[1]
Culture	+	n/a	+	–	+	–
Staining	+	n/a	+	+	+	–
Genotyping	n/a	n/a	n/a	n/a	++	n/a
Detection of immune response						
ELISA	+++	n/a	+++	++	+++	+++
IFA	++	n/a	++	++	++	++
CFT	–	n/a	–	++	+	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limit its application; – = not appropriate for this purpose; n/a = not applicable.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

2.9.1 Detection of the organism

The isolation of *C. burnetii* should be done in biosafety level 3 laboratories only due to its high infectivity. This microorganism can be isolated by inoculation of specimens onto conventional cell cultures or into embryonated hen egg yolk sacs or laboratory animals, such as mice or guinea pigs (Ormsbee, 1952; Williams et al.,1986). The culturing of *C. burnetii* is a slow and unreliable method for detecting *C. burnetii*, but has been used in some experiments. Microscopic examination of *C. burnetii* organisms in placental tissues is done using Stamp-Macchiavello staining in which heat-fixed smears are stained with basic fuchsin, before

decolourisation with citric acid and counter-staining with methylene blue (Bildfell et al., 2000). A Gimenez stain is also often used (Gimenez, 1964). Microscopic examination of stained tissues for *C. burnetii* detection has a poor specificity because *C. burnetii* can be confused with other organisms like *Chlamydia spp* and *Rickettsia spp.* (Porter et al., 2011).

Immunohistochemistry (IHC) is a very promising tool for the diagnosis of ruminant coxiellosis and it can be utilized for detection of bacteria in tissues fixed in paraffin (Figure 4). It can contribute to a better understanding of reproductive disorders in cattle through evaluation of endometrial and placental biopsy samples (de Biase et al., 2018).

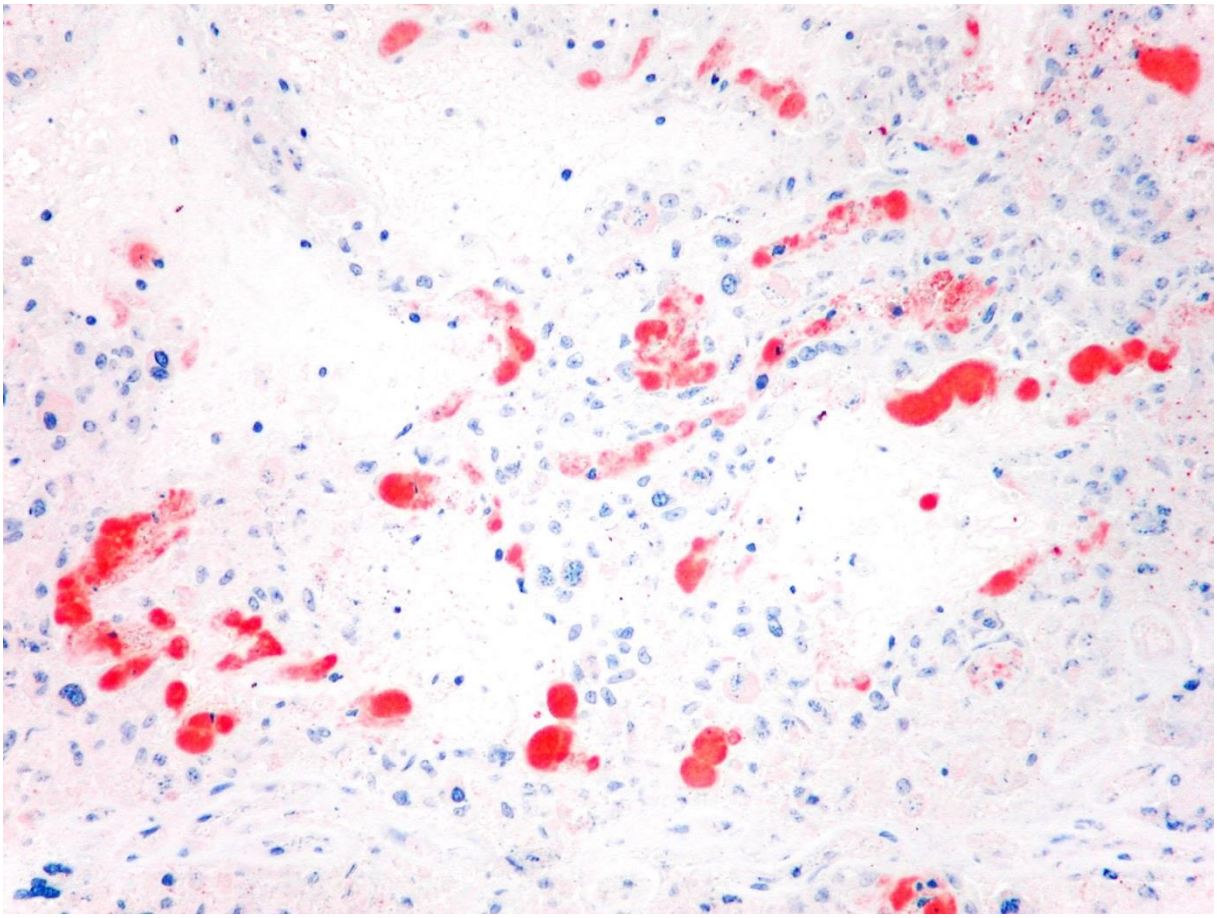

Figure 4. Section of a goat cotyledon. *C. burnetii* are seen in large cytoplasmic inclusions in the trophoblast cells, and less frequently on the surface of the layer of trophoblast cells. Labelled streptavidin-biotin method, counterstaining with Mayer's haematoxylin. × 200 (L. Szeredi)

2.9.2 Serological methods

Antibodies are produced within a short timeframe of usually 2 to 3 weeks after infection with *C. burnetii* in animals (Roest et al., 2013). Antibodies to *C. burnetii* in ruminants and humans have also been reported to remain in circulation for long periods, thus making serological diagnosis a reliable method of detecting exposure. Serological diagnosis of Q fever in the early stage of infection can be unsuccessful due to the timeframe of seroconversion spanning 2-3 weeks post infection (Howe and Mallawia., 2000; Teunis et al., 2013). Serological analyses may be carried out using ELISA, IFA or CFT. IFA is the reference method used mostly in human medicine. While CFT and ELISA are also used in humans, they are the serological methods most frequently used for routine diagnosis of Q fever in animals (Niemczuk et al., 2011). The advantage of ELISA is that it is easy to perform, interpretation is less subjective than for IFA and CFT, and automation is possible (Eldin et al 2017).

CFT was the first serological method used for detecting *C. burnetii* antibodies. The CFT was based on the protocol described in the World Organization for Animal Health (OIE) Manual of Standards and had been in routine use by most of the national reference laboratories in the past 30-40 years. CFT detects both Phase I and Phase II antibodies. Antibody concentrations are expressed as titers of between 1:10 and >1:640 using doubling dilutions. The OIE guidelines state that a titer of ≥1:80 is significant and indicates recent infection, titers between 1:10 and 1:40 represent a latent infection and a titer <1:10 is negative. This assay is being widely reported to have a very low diagnostic sensitivity and non-specific reactions on some samples leading to uninterpretable results (Rousset et al., 2007; Niemczuk et al., 2011). A number of studies have reported that IFA and indirect ELISA are more sensitive than CFT for diagnosis of coxiellosis in ruminants, however it is a useful method to differentiate acute and chronic infections (Kittelberger et al., 2009; Niemczuk et al., 2011; Horigan et al., 2011; Szymanska et al., 2013).

Indirect ELISA is a sensitive and specific alternative for the diagnosis of Q fever in ruminants. Moreover, the ELISA method is also suitable for evaluating the prevalence of *C. burnetii* in herds in serological surveys (OIE, 2018). Testing bulk tank milk or pooled individual samples by ELISA can be used for prevalence estimation, but must be assessed in relationship to the intra-herd prevalence (OIE, 2018). ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and it is the most robust method. Several ready-to use ELISA kits are commercially available and can detect mixtures of anti-Phase I and II antibodies. All kits use a conjugate that detects specific ruminant IgG antibodies to provide evidence of exposure to *C. burnetii* infection. The results are based on optical density (OD) and expressed as sample OD/positive control OD × 100 = sample-to-positive (S/P) ratio. As detailed in the individual test kits, there are slight variations in the way

S/P ratios are calculated between manufacturers to account for the background OD of the sample or control. Since the first description of an ELISA for the detection of *C. burnetii*-specific IgM, this method has become a frequently-used method for seroepidemiological surveys of Q fever (Field et al.,1983).

IFA is the human reference method and has been reported to have a diagnostic sensitivity ranging from 98% to 100%, and a diagnostic specificity of 95% for human sera (Fournier et al.,1998; Meekelenkamp et al., 2012). IFA is the most sensitive technique for detecting IgM antibodies at an early phase of infection and after 12 months of follow-up. Regarding IgG, IFA was more frequently positive than ELISA and CFT (100%, 95.2%, and 96.8%, respectively) (Wegdam-Blans et al., 2012). Even though several IFA tests are available commercially, most reference laboratories have developed their own in-house immunofluorescence assay. Both Phase I and Phase II *C. burnetii* antigens are used in all IFA methods. Phase II antigens are obtained by growing the *C. burnetii* Nine Mile reference strain in a cell culture, while Phase I antigens are obtained from the spleens of laboratory animals. The antigen is diluted, dropped onto the wells of a glass microscope slide, allowed to dry, and fixed with acetone. In humans the acute and chronic forms of the infection have different serological profiles. During acute Q fever, only IgG antibodies against Phase II antigens are elevated, whereas during chronic Q fever, high levels of IgG antibodies against both Phase I and II of the bacteria are observed (Tissot-Dupont et al., 1999).

The main diagnostic specifications published for ELISA, IFA and CFT are presented in Table 2., all showing that CFT is less sensitive than ELISA or IFA, despite the test being standardised across laboratories and not being species-specific.

Table 1. Previously published diagnostic sensitivities and specificities of ELISA, IFA and CFT

Species	Methods	Test/ (antibody)	Diagnostic sensitivity %	Diagnostic specificity %	Ref.
Cattle, sheep and goats	Relative comparison with the ELISA (presumed gold standard)	CFT (Cattle) CFT(sheep and goats)	26.6 10.0	99.7 99.9	Natale et al., 2012
Cattle and goats	Using infected and noninfected samples	aI-ELISA bP-ELISA CFT	95 81 68	100 99 100	Kittelberger et al., 2009
Cattle, sheep, goats	Receiver Operating Characteristic curve, maximum likelihood methods	cELISA 1 dELISA 2 eELISA 3 CFT	87.0 98.6 55.7 36.2	99.1 97.1 99.3 98.3	Horigan et al., 2011
Human	Using infected and noninfected samples	ELISA CFT	98.6 72.9	87.6 89.9	Field et al., 2000
Human	Using infected and noninfected samples	IFA (IgM Phase II.) ELISA (IgM Phase I.)	100 85.7	95.3 97.6	Meekelenkamp et al., 2012
Human	Used ELISA as reference	IFA (IgG phase II.) IFA (IgG phase I.)	97.7 87.2	100 90	Slaba et al., 2005

The types of ELISA kits used were from a: IDEXX, United States of America, b: Institute Pourquier, France, c: had ovine derived antigen, d: had tick derived Nine Mile antigen, e: had bovine derived antigen.

2.9.3 Genomic detection of *C. burnetii*

Polymerase chain reaction (PCR) is used for the detection of DNA of the organism in tissues like placenta and in secretions like birth fluids and milk (OIE, 2018). These reactions target DNA sequences known to exist in the *C. burnetii* genome and considered to be absent from the genomes of other organisms. Some of the *Coxiella* genome sequences that have been targeted by PCR reactions include the highly conserved single copy *com1* and *htpB*, plasmid *QpH1* and *QpRs* genes as well as the multiple copy transposase *IS1111* element (Klee et al., 2006 Harris et al., 2000). PCR methods of detecting *C. burnetii* DNA are considered to be highly sensitive and sufficiently informative for the diagnosis of Q fever (Sidi-Boumedine et al., 2010; Malou et al., 2012). PCR assays targeting the multi-copy genes as *IS1111* are important in detecting C. *burnetii* but may be limited for quantifying the concentration of *C. burnetii* present in the original samples, whereas single-copy genes like *com1* are important in

quantifying the number of *C. burnetii* organisms present as every copy of the gene detected corresponds to a single organism (Lockhart et al., 2011). Real-time PCR provides an additional means of detection and quantification (Klee et al., 2006). In contrast to conventional PCRs, where various target genes are used, for real-time PCR it is recommended to amplify a unique and specific sequence. Several ready-to-use PCR kits are commercially available and can detect the causative agent in various sample types. PCR has been shown to detect *C. burnetii* DNA in peripheral blood cells within days of exposure in humans. There is a 2 to 3 week window following infection without seroconversion, until antibodies can be detected in blood samples (Roest et al., 2013; Wielders et al., 2013). In one experimental infection of goats with *C. burnetii*, the earliest PCR positive blood samples were obtained 28 days after exposure, much later after antibodies to *C. burnetii* were detected (Roest et al., 2012).

2.9.4 Genotyping methods

Genotyping of *C. burnetii* is a key tool in understanding the epidemiology of Q fever. As Q fever is a zoonosis, it is important to find the possible animal sources of human outbreaks. Although several genotyping systems exist, two PCR-based typing methods have been most frequently used recently: MST and multi-locus variable-number tandem repeat analysis (MLVA) (Glazunova et al., 2005; Arricau-Bouvery et al., 2006; Svraka et al., 2006). MST was introduced by Glazunova et al., who identified 10 highly variable spacers located between 2 open reading frames (ORFs) (Glazunova et al., 2005). This typing method identified 30 different genotypes and three monophyletic groups among 173 *C. burnetii* isolates. This method is very discriminant and has been used most frequently in different studies around the world. MST genotyping helps to trace the spread of *C. burnetii* from one region to another and from animal reservoirs to humans. According to Eldin et al., this genotyping has been qualified as a “geotyping” method (Eldin et al., 2017). This “geotyping” scheme is still incomplete and has to be implemented in further studies to provide an overall map of the genetic diversity of *C. burnetii*.

MLVA genotyping was established by Svraka et al., who amplified a multiple locus variable number tandem repeats (VNTR) sequences from 21 *C. burnetii* isolates (Svraka et al., 2006). Although MLVA is based on the analysis of relatively unstable repetitive DNA elements, this method has a high discriminatory power. Furthermore, it significantly lacks interlaboratory reproducibility (van Belkum 2007).

2.10 Treatment and Control

In human medicine it is recommended to initiate antibiotic treatment using doxycycline (200 mg per day) (Eldin et al., 2017). During the biggest Q fever outbreak all over the world, which was in the Netherlands, it was confirmed that treatment with doxycycline, a fluoroquinolone, clarithromycin, or co-trimoxazole was associated with a reduced risk of hospitalization compared with that for patients receiving beta-lactams or azithromycin (Dijkstra et al., 2011). Many studies indicate that a delay in diagnosis and treatment was associated with a higher rate of hospitalization and development of chronic infections and secondary complications (Porter et al., 2011.; Eldin et al., 2017).

In livestock animals, especially in lactating dairy cattle, the use of tetracyclin is not allowed. In general, it is rare to use antibiotics to treat bovine coxiellosis. With Q fever being a zoonosis, prophylaxis at herd level is fundamental to limit disease transmission both to humans and at the herd level. Preventive veterinary and standard hygiene measures are key in the control of Q fever in livestock. Proper manure management is also of key importance to avoid spreading bacteria from infected farms to the environment. The strictness of control methods vary by country. In France, when Q fever is diagnosed in a herd on a cheese-producing farm, milk of the aborted females must be discarded. Sale, processing and treatment of this milk is strictly forbidden for one year after the initial diagnosis of disease in an animal (AFSSA, 2007). Control strategy in the Netherlands is focused on vaccination against *C. burnetii,* it became compulsory in all goats and sheep and dairy farms in the south of the country, mainly in the province of Noord-Brabant (Dijkstra et al., 2012).

2.11 Vaccination

Currently prophylaxis includes vaccination with the nonfully licensed inactivated Phase I vaccine, Coxevac (CEVA-Sant´e Animale, Libourne, France), when a focus of Q fever is declared. The active substance of the Coxevac vaccine is a Phase I, Nine Mile (strain RSA 493) *C. burnetii* strain, formalin-inactivated, providing an inactivated bacterial vaccine for cattle and goats. The use of the Nine Mile strain (Phase I) as a vaccine strain is recommended by OIE. *C. burnetii* has 2 antigenic forms, called Phase I and Phase II. The bacteria in Phase I have longer lipopolysaccharide chains on their surface than those in Phase II, and thereby have different antigenic properties. Phase I is the infective form and is found under natural circumstances. Phase II exists only under laboratory conditions, after serial passages on embryonated eggs or cell culture. Administration of Phase II antigens induces the production of antibodies against Phase II antigen only, whereas vaccination with Phase I antigens elicits

the production of antibodies against both Phase I and Phase II antigens. The major Phase I specific antigen is Phase I LPS. There are other proteins which can be different between Phase I and Phase II but these are not considered as important for inducing protective immune responses. Phase I LPS is the main antigen which is responsible for the protection induced by vaccination with inactivated Phase I bacteria. Another animal vaccine, Chlamyvax-FQ (Merial Inc., Lyon, France), a Phase II. *C. burnetii*, was commercially available in France; this was shown not to be efficacious, presumably because it contained only Phase II. antigens (Arricau-Bouvery et al., 2005). Vaccination of ruminants with inactivated Phase I *C. burnetii* antigens one month before breeding is the most commonly used strategy of controlling *C. burnetii* in infected domestic ruminant herds, as recommended by the manufacturers of the only existing livestock vaccine, Coxevac (Hogerwerf et al., 2011; Rousset et al., 2009; Astobiza et al., 2011; Pinero et al., 2014; Taurel et al., 2014; Guatteo et al., 2008). The goal of vaccination against *C. burnetii* in livestock has previously been described as to reduce environmental contamination by infected livestock and to consequently reduce the risk of infection in humans and animals. Rousset et al. tested the efficacy of a Phase I *C. burnetii* vaccine administered before breeding and they found a lower proportion (4%) of vaccinated sheep and goats among high shedders compared to 13% of non-vaccinated sheep and goats being identified as high shedders (Rousset et al., 2009). Vaccination of livestock before breeding has been shown to reduce *C. burnetii* shedding and *C. burnetii*-associated abortions in infected herds (Rousset et al., 2009; Astobiza et al., 2011; Taurel et al., 2014; Cremoux et al., 2012). Vaccination against Q fever is a medium-long term strategy in dairy cattle farms. The progression of *C. burnetii* infection after implementing a two-year vaccination program in a naturally infected dairy cattle herd was published by Pinero et al (Pinero et al., 2014). This research found that individual milk samples showed a gradual decline in the percentage of *C. burnetii* milk shedders throughout the study period. Before vaccination, 9.0% of lactating cows were milk shedders and this prevalence gradually decreased to 1.2% within two years. No shedders were detected among younger milking cows after vaccination (Pinero et al., 2014). Another study highlighted that cattle vaccinated while not pregnant, had a five-fold lower probability of becoming a shedder. Thus susceptible animals, especially heifers should be vaccinated, if it is possible (Guatteo et al., 2008). Vaccination with Phase I *C. burnetii* vaccine improved some of the reproductive parameters in high producing lactating cows in *Coxiella*-infected herds. According to this study in animals testing seronegative for *C. burnetii,* the likelihood of pregnancy was 1.25 times higher in vaccinated cows compared to non-vaccinated seronegative, thus vaccination improves subsequent fertility of *C. burnetii* seronegative animals (López-Helguera et al., 2013). Similar research stated that two consecutive vaccination rounds against *C. burnetii* in advanced gestation reduce subfertility and early fetal loss in dairy cows (Garcia-Ispierto et al., 2015). A significant reduction in *C. burnetii* load was found in herds where a

vaccination of ≥80% of dairy cows was implemented (Taurel et al., 2014). The affinity of the bacterium for trophoblast cells of the placenta and the enormous replication of *C. burnetii* in the trophoblasts would be expected to limit the efficacy of vaccination in pregnant livestock. This hypothesis is supported by the findings of many studies that have shown vaccination of pregnant animals not to be effective in reducing the proportion of shedders and the load of *C. burnetii* shed. In dairy cattle, the proportion of vaccinated non-pregnant heifers and vaccinated non-pregnant cows shedding *C. burnetii* was lower than the proportion of shedders in both vaccinated pregnant heifers and vaccinated pregnant cows (Taurel et al., 2014). These proportions of vaccinated pregnant cattle shedding *C. burnetii* were not statistically significantly different from those observed in unvaccinated heifers and cows, which further highlights the lack of efficacy of the Coxevac vaccine when administered in pregnant cattle in infected herds (Taurel et al., 2014). These results should be taken into account when developing the most effective vaccination strategy in *C. burnetii* in infected dairy herds.

3. Aims of the study

The aims of the study were:

1. To assess the prevalence of *C. burnetii* in dairy cattle herds of different sizes in six countries of Central and Eastern Europe, examining bulk tank milk samples with ELISA and real-time PCR tests.
2. To evaluate the prevalence of *C. burnetii* antibodies in different hosts (dairy cattle, sheep, goats, and zoo animals) in Hungary
3. To determine the importance of Q fever in dairy cattle farms as a zoonotic risk factor and to estimate the seroprevalence of *C. burnetii* in different occupational groups of farm workers and to compare Q fever infection rate in farm veterinarians in different countries based on the presence of IgG to Phase I and Phase II antigens of *C. burnetii*.
4. To determine the effect of C. *burnetii* seropositivity by ELISA and by the complement fixation test (CFT) in the early pregnancy diagnosis and pregnancy losses in dairy cows between days 29 and 70 of gestation in some Hungarian dairy herds.
5. To compare the occurrence of *C. burnetii* in retained fetal membranes and normally separated placentas and to reveal of importance of C*. burnetii* in retention of fetal membranes (RFM) in dairy cattles.
6. To compare the genotypes of *C. burnetii* using the MST assay from Hungary and Slovakia.

4. Materials and methods

4.1 Samples

4.1.1 Bulk tank milk samples

Bulk tank milk samples were collected from 370 dairy herds from six Central and Eastern European countries (Croatia, n=13; Czech Republic, n=138; Hungary, n=126; Serbia, n=24; Slovakia, n=53; Slovenia, n=16) between March and October 2019 (Table S1; Figure 5). Samples were taken randomly from dairy herds of different sizes, but with focusing on larger dairies.

Forty ml samples were taken from each bulk milk tanks.

Figure 5. Geographical distribution of the tested 370 dairy herds in Central and Eastern Europe (Croatia, Czech Republik, Hungary, Serbia, Slovakia, Slovenia) between March and October, 2019.

4.1.2 Blood samples from different animal species

Blood samples were collected between May 2019 and December 2020 from three large statistical geographic regions of Hungary (Transdanubia, Great Plain and North). A total of 851 serum samples were tested from 44 dairy farms, 16 sheep flocks, four goat farms and three zoos (Figure 6). Samples from zoo animals were also collected in the Central region but not selected from other species as that region is industrial. The herds and flocks included in the study were based on the following criteria: farm size above 350 animals, use of regurarly updated farm records and willingness to provide data. Participation in the study was voluntary and we encouraged farmers and veterinarians to sample the animals with suspected Q fever because of infertility or a previous diagnosis of abortion, premature delivery or stillbirth. There were no special inclusion criteria for zoo animals, and the objective was to include as many ungulate species as possible. Seropositivity to *C. burnetii* was surveyed in dairy cattle (n=547), goats (n=71), sheep (n=200) and zoo animals (n=33), among them different wild ungulate species including camels, alpacas, bison, Cameroon goats, fallow deers, giraffes, antelopes, reindeer, and buffaloes.

Figure 6. Geographical distribution and *C. burnetii* ELISA status of the dairy cattle herds, sheep flocks, goat herds and zoos surveyed in Hungary between May 2019 and December 2020.

4.1.3 Blood serum collection associated with early pregnancy loss

Data and blood were collected in October and November 2019 from all inseminated cows of three Hungarian dairy farms (herd size: 600, 750 and 1,000 cows, milk production: 9,600, 10,200 and 11,000 kg/cow/year, respectively).

All cows contributing to the data set were Holstein-Friesian, fed a total mixed ration (TMR) and bred by artificial insemination (AI) after a voluntary waiting period of ~60 days. Pregnancy status was determined by the measurement of serum pregnancy-specific protein B (PSPB) concentrations (29-35 days after AI; n = 321). In all cows initially designated pregnant, continuation of pregnancy or pregnancy loss was determined by transrectal palpation 60-70 days after AI. At 29-35 days after insemination, a blood sample from the coccygeal vein of each cow was collected and sent to the laboratory by overnight mail. Upon arrival at the laboratory, blood samples were centrifuged (670 × g for 10 min) and the resulting sera were assayed for PSPB (BioPRYN^{TM}; Biotracking, Moscow, ID, USA), as described previously (Gábor et al., 2007). Next day the serum samples were sent to the laboratory for serological testing.

4.1.4 Human blood samples

Human blood samples collected from 70 dairy farm workers between February and July 2020 were tested for the presence of antibodies to *C. burnetii*. The study was approved by the Hungarian Scientific Ethics Committee and all subjects provided their informed consent. The eight dairy cattle farms included in the survey are located in different parts of Hungary, equally distributed between three large statistical geographic regions of the country (Transdanubia, Great Plain and North). All dairy units had between 600 and 1,000 milking cows, which had previously been found to be ELISA and PCR positive for *C. burnetii* by bulk tank milk testing. Industrial dairy farms were included in this study based on willingness to provide a human blood sample and data from all workers to the authors. Participation in the study was voluntary and we encouraged farms to participate in this research. The five occupational groups were categorized into three risk groups based on their possible close contact with *C. burnetii* infected animals, mostly placenta and other birth products. The examined group consisted of veterinarians (n=8), inseminators (n=12), as a high-risk occupational group, animal caretakers (n=26), as a medium risk group and herd managers (n=7) and milking parlour workers (n=17) as a lower risk group. The population under study consisted of 13 women and 57 men, aged between 19 and 64 years. Questionnaires were used to record the participants' demographic data, occupation, length of employment at the farm, and any

symptoms of a potential previous Q fever (any fever with headache, pneumonia, myalgia, hepatitis, swollen lymphnodes) (Table S3).
Blood samples also were collected between May and September 2020 from 19 Hungarian and 5 Slovakian veterinarians working on large industrial dairy cattle farms.

4.1.5 Bovine placenta samples from Hungary and Slovakia

Cotyledons were collected from randomly selected cows after parturition between June 2019 and November 2020 in 30 Hungarian and 5 Slovakian dairy herds. The size of the herds ranged between 600 and 1,500 animals. All tested cattle belonged to the Holstein-Friesian breed. A total of 167 cotyledons from Hungary (n=157) and Slovakia (n=10) were sampled, 77 of which were collected from normally calving cows and 90 from cows with delayed placental separation of more than 12 h after expulsion of the fetus (LeBlanc, 2008). The farm veterinarians selected one cotyledon per placenta which was stored at -19 °C on the farms.

4.2 Sample processing

4.2.1 Lactoserum processing

Lactoserum was separeted from the milk samples for ELISA tests by two-step centrifugation, consisting of centrifugation at 3000 × g for 20 min at 4°C, and from 1 ml supernatant (pipetted from under the ring of milk fat) centrifugation at 7000 × g for 15 min.
Somatic cells from milk samples were concentrated using low-speed centrifugation (3000 x g for 20 min at 4°C), then 1 ml cell pellets were further centrifuged at 12 000 × g for 10 min at 4 °C. DNA extraction from 200 µl of the gained cell pellets was performed using the Qiagen DNA Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

4.2.2 Placenta processing

Cotyledons were sliced up and mixed with 10 mL of phosphate-buffered saline, then homogenised with a laboratory blender. Twohundred microlitre sediments of centrifuged (12 000 × g for 10 min at 4°C) homogenates were subjected to DNA extraction with the Qiagen DNA Mini kit (Qiagen Inc.) according to the manufacturer's instructions.

4.3 Enzyme-linked immunosorbent assay

Lactosera were tested with commercial ELISA kits (ID Screen® Q Fever Indirect Multi-species, IDVet Inc., Grabels, France; IDEXX Q Fever Ab Test, IDEXX Europe B.V., Hoofddorp, the Netherlands) according to the manufacturers' instructions. Interpretation of the results gained by the ID Screen® Q Fever Indirect Multi-species kit was based on the evaluation of S/P % values, considering positive the samples with S/P % > 20 in case of the diluted bulk tank milk samples (1:50 dilution). If diluted samples showed negative results, the undiluted samples were tested also, considering negative the samples with S/P % ≤ 30%, positive the samples with S/P % > 40% and doubtful the samples with 30% < S/P % ≤ 40%. Interpretation of the results gained by the IDEXX Q Fever Ab Test kit were based also on the evaluation of S/P % values, considering positive the samples with S/P % > 30, examining diluted bulk tank milk samples (1:5 dilution).

The blood samples were tested with a commercial ELISA kit (ID Screen® Q Fever Indirect Multispecies, IDVet Inc.) used according to the manufacturer's instructions. Serum samples diluted 1:50 were used in the ELISA microplate. 100 µl pre-diluted negative, positive controls were used, and same 100 µl of each pre-diluted samples were tested in remaining wells. After 45 minutes of incubation at 21°C each well was washed 3 times with 300 µl wash solution. 100 µl conjugate was added to each well, the plate was covered and incubated for 30 minutes at 21°C. After incubation the wells were washed again and 100 µl substrate solution was added. After the final incubation 100 µl stop solution was added to stop the reaction. Optical density (OD) value was read and recorded in 450nm. The test was considered valid if the positive control OD was greater 0,350 and the ratio of the mean values of the positive control OD to the negative control OD is greater than 3. Interpretation of the results gained by the IDEXX Q Fever Ab Test kit were based also on the evaluation of S/P % values, considering negative the samples with ≤ 30%, doubtful 40% <S/P % ≤ 50%, positive 50% <S/P % ≤ 80% and strongly positive S/P % > 80%.

4.6 Complement fixation tests

The ELISA positive serum samples were further examined with two different CFT tests, using *C. burnetii* Phase I and II antigens, according to the manufacturer's instructions (Virion/Serion GmbH, Würzburg, Germany), and the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Word Organisation for Animal Health, 2018). The reaction was done in two stages. Antigen and complement-fixing antibodies were first mixed and incubated overnight at 4 °C. Sheep erythrocytes sensitised by the anti-sheep erythrocyte serum were added the next day.

Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum. We made twofold dilutions of inactivated sample sera from 1/10 to 1/320 in six wells, and dilutions from 1/10 to 1/80 in four additional wells to detect anticomplementary activity (25 µl per well). Twentyfive µl of diluted antigen or 25 µl of Veronal/calcium/magnesium buffer (VB) was added to control serum wells, while 25 µl of diluted complement was added to all wells. The plate was covered with plastic adhesive film and incubated for 18 hours at 4°C. The plates were removed from the refrigerator and allowed to reach room temperature, adding 25 µl of the freshly prepared haemolytic system. The plates were then incubated at 37°C for 30 minutes and centrifuged at 500 g for 5 minutes at 4°C. Finally, we examined the controls and read the results. Interpretation of the results: Titres between 1/10 and 1/40 are characteristic of a latent infection and titres of 1/80 or above were considered characteristic of an active phase of the infection.

4.5 Immunofluorescence assay

Human serum samples were tested for the presence of IgG reacting with Phase I and Phase II antigens of *C. burnetii* strain Nine Mile using a commercially available immunofluorescence assay (Focus Diagnostics, Cypress, CA). The test was performed according to the manufacturer's instructions in a reference laboratory at the National Public Health Centre, Budapest, Hungary. Cutoff value was set at serum-dilutions 1:16 as a screening procedure, with end-titres determined for seropositive samples.

4.6 Polimerase chain reaction for the detection of *C. burnetii*

A fragment of the IS*1111* the transposase gene was amplified using a real-time PCR system with the following primers and TaqMan probe: IS1111F: CCGATCATTTGGGCGCT, IS1111R: CGGCGGTGTTTAGGC and IS1111P: 6FAM-TTAACACGCCAAGAAACGTATCGCTGTG-MGB (Loftis et al., 2006). PCR was performed in 12,5 µl total volume, containing 1 µl target DNA, 6.775 µl commercially purified water (Millipore Co., Billerica, MA), 1.25 µl AmpliTaq Gold buffer (Applied Biosystems Inc., Foster City, CA), 1.25 µl $MgCl_2$ (25 mM; Thermo-Fisher Scienctific Inc., Waltham, MA), 0.5 µl dNTP (10 mM; Thermo-Fisher Scienctific Inc.), 0.5 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 0.125 µl of probe (10 pmol/µl) and 0.1 µl AmpliTaq Gold polymerase (5 unit/µl; Applied Biosystems Inc.). PCR amplifications

were performed on a CFX96 Touch Real-Time PCR instrument (Bio-Rad Inc., Hercules, CA). The PCR consisted of initial denaturation for 10 min at 95 ºC followed by 45 amplification cycles of denaturation for 15 sec at 95 ºC and primer annealing and extension at 60 ºC for 1 min.
The detection threshold of the PCR system was ~0.1 CFU (Ct 36.95), according to a commercially available positive control (Adiavet Cox, Aes Chemunex Inc., Cranbury, NJ) (Table 3).

Table 3. Mean Ct value and threshold of the PCR system according to a commercially available positive control (Adiavet Cox; Aes Chemunex Inc., Cranbury, NJ, USA)

CFU/µl	Mean Ct value
1000	23.68
100	27.08
10	30.82
1	33.60
0.1	36.95
0.01	negative

4.7 Multispacer sequence typing

For the MST analysis ten selected spacer regions (Cox 2, 5, 6, 18, 20, 22, 37, 51, 56 and 57) of the *C. burnetii* genome were amplified and sequenced as described by Glazunova et al. (2005). The primer pairs, listed in Table 4 were used.

Table 4: Primers used for PCR amplification and sequencing of *C. burnetii* gene spacers (Glazunova et al., 2005)

Spacer name	Open reading frame	Nucleotide sequence (5´–3´)	Amplified fragment length (bp)
Cox2	Hypothetical protein	Cox20766 CAACCCTGAATACCCAAGGA	397
	Hypothetical protein	Cox21004 GAAGCTTCTGATAGGCGGGA	
Cox5	Sulfatase domain protein	Cox77554 CAGGAGCAAGCTTGAATGCG	395
	Entericidin, putative	Cox77808 TGGTATGACAACCCGTCATG	
Cox18	Ribonuclease H	Cox283060 CGCAGACGAATTAGCCAATC	557
	DNA polymerase III, epsilon subunit	Cox283490 TTCGATGATCCGATGGCCTT	
Cox20	Hypothetical protein	Cox365301 GATATTTATCAGCGTCAAAGCAA	631
	Hypothetical protein	Cox365803 TCTATTATTGCAATGCAAGTGG	
Cox22	Hypothetical protein	Cox378718 GGGAATAAGAGAGTTAGCTCA	383
	Amino acid permease family protein	Cox378965 CGCAAATTTCGGCACAGACC	
Cox37	Hypothetical protein	Cox657471 GGCTTGTCTGGTGTAACTGT	463
	Hypothetical protein	Cox657794 ATTCCGGGACCTTCGTTAAC	

The PCR mixtures contained 5 µL 5×Green GoTaq Flexi buffer (Promega Inc., Madison, WI), 2.5 µL $MgCl_2$ (25 mM; Promega), 0.5 µL dNTP (10 mM; Thermo-Fisher Scientific Inc.), 2 µL of each primer (10 pmol/µl), 0.25 µL GoTaq Flexi Polymerase (5U/µl, Promega) and 2 µL DNA template with a total volume of 25 µL. The PCR was performed on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc.). Thermocycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 57 °C for 30 sec and 72 °C for 1 min. After

amplification the reaction mixture was subjected to electrophoresis in 1% agarose gel, and the amplified gene products were visualized with ultra violet light after GR Safe nucleic acid gel staining (Lab Supply Malla InnoVita Inc., Gaithersburg, MD). PCR products were isolated from agarose gel with the QIAquick Gel Extraction Kit (Qiagen Inc.) and sequenced on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems Inc.).
For the analysis the sequences of the gene fragments were concatenated. To determine the sequence types (STs), an alignment comparison with the sequences in the MST Database (https://ifr48. timone.univ-mrs.fr/mst/coxiella_burnetii/strains.html) was performed. Phylogeny was inferred by using the Neighbourjoining method and Tamura 3-parameter model estimated in MEGA X software (Tamura, 1992; Kumar et al., 2018).

4.8 Immunohistochemistry

An immunohistochemical method (IHC) was used to detect *C. burnetii*, in formalin-fixed and paraffin-embedded tissue samples of fetal membranes containing histological lesions. After dewaxing the sections, antigen retrieval was performed in citrate buffer (pH 6.0), by heating in a microwave oven (750 W) for 20 min. The samples were incubated in 3% H_2O_2 solution for 10 min and then a blocking step was performed with a 2% solution of skimmed milk powder for 20 min. The sections were incubated with the primary antibodies at 4 °C overnight at a dilution of 1:2000 (anti *C. burnetii* antibody, provided by Ceva-Phylaxia). Antibody binding was detected with a horseradish-peroxidase labelled streptavidin-biotin kit according to the manufacturer's instructions (Universal LSAB2 Kit-HRP, Dako Co., Glostrup, Denmark). The sections were treated with 3-amino-9-ethylcarbazole solution (Sigma-Aldrich Co., St Louis. MI) also containing 0.01% H2O2, at room temperature for 10 min, counterstained with Mayer's haematoxylin for 20 sec, and covered with glycerol-gelatine. Tissue sections infected with the corresponding agent were used as positive controls. Immunohistochemistry was used to detect the functional changes induced by *C. burnetii* in the placenta.

4.9. Statistical analysis

4.9.1 Statistical analysis of bulk milk results

Spearman's rank correlation was applied to analyze correlation between infection status (percentage of all positive test results by ELISA and PCR) and herd size (ranked as follows: 1= herd size of 50-249 animals, 2= 250-499 animals, 3= 500-999 animals, and 4= herd size of ≥1000 animals), using R software (R-core Team, 2020).

4.9.2 Statistical analysis for the serology of different host species

Cattle, goat, and sheep farms were considered positive if at least one animal was tested ELISA positive. The occurrence of seropositivity on animal level was compared among cattle, small ruminants (i.e., sheep and goats grouped together), and zoo animals using Fisher's exact test. P values were corrected for multiple comparisons using False Discovery Rate correction. Furthermore, the odds of seropositivity on animal level were modelled, taking the geographical region into account, in those groups of animals where at least one positive animal was found. For this purpose, a logistic mixed model was built with seropositivity as a binary dependent variable, animal type and geographic region as fixed factors, and farm as random effect, using the glmmTMB package (Brooks et al., 2017). Statistical analysis was performed in R 4.0.3. (R Core Team, 2020).

4.9.3 Statistical analysis of human blood sample results

Risk groups were compared by pairwise Fisher's exact tests. P-values were adjusted for multiple comparisons using FDR correction. The level of significance was set to 0.05. Statistical analysis was performed in R 4.0.2 (R Core Team, 2020).

4.9.4 Statistical method used for the analysis of early pregnancy loss

All 321 samples were examined by ELISA and all the ELISA-positive animals were further examined by CFT, using *C. burnetii* Phase I and II antigens. Commercial ELISA kits (ID Screen® Q Fever Indirect Multi-species, IDVet Inc.) were used according to the manufacturers' instructions. The serum samples were examined by two different CFT tests, utilising *C. burnetii* Phase I and II antigens, according to the manufacturer's instructions (Virion/Serion GmbH, Würzburg, Germany), and the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Word Organisation for Animal Health, 2018).

The percentage pregnancy loss (PPL) was calculated as follows: the calculation used the number of cows diagnosed pregnant at 29-35 days after AI (based on PSPB concentration) and the number of cows diagnosed pregnant by transrectal palpation 60-70 days after AI.

Spearman's rank correlation was applied to analyse the correlation between the positive ELISA test results and the number of pregnancies (ranked as follows: 1 = positive test result, 0 = negative test result. Pregnancy loss was represented with rank 1 and continuous pregnancy was ranked 0), using R software (R Core Development Team, 2020).

A dataset was created based on the results of the ELISA and CFT results, where a pregnancy lost in Phase I was marked with 1 and pregnancies lost were marked with 0. Similarly, the cows that lost a pregnancy in Phase II were marked with 1 and animals that were pregnant in Phase II were marked with 0. The means of the two datasets (pregnant animals in Phase I and II and pregnancy loss in Phase I and II) were compared with Student's *t*-test, using R software (R Core Development Team, 2020).

4.9.5 Statistical method used for the analysis bovine placentas

The relationship between the occurrence of retained placenta and the presence of *C. burnetii* in the cotyledon samples as indicated by PCR was examined by multivariate mixed-effects logistic regression. The occurrence of retained placenta was a binary dependent variable (yes/no), whereas the *C. burnetii* PCR result (positive/negative), parity category (Parity 1, Parity 2, Parity 3+) and their interaction were included in the initial model as explanatory variables. The farm was the random effect. The interaction term was not significant; therefore, it was removed from the final model. Model building was performed using the glmmTMB package in R (Brooks et al., 2017). Multiple comparisons were performed by Tukey's *post-hoc* test using the multcomp package in R (Hothorn et al., 2008). The explanatory variables were tested for collinearity using the variance inflation factor (VIF): VIF larger than 2.5 was indicative of collinearity in this study. No collinearity was detected. Statistical analyses were performed in R version 4.0.5 (R Core Team, 2020).

5. Results

5.1. Results of the ELISA tests and PCR assays of the bulk tank milk samples

In Hungary 4 out of the 7 (57.14%) examined herds showed positivity (2 ELISA and 2 both ELISA and PCR) in the category 50-249 milking cows. All bulk tank milk samples were positive in the other three herd size categories (250-499; 500-999; ≥1000). Seventy-four herds were just ELISA positive and 45 both ELISA and PCR. Only PCR positivity was not found without ELISA positivity in any of the samples. In Slovakia the figures have shown the same distribution as in Hungary. Fifteen out of the 20 (75.0%) examined herds showed positivity (5 ELISA and 10 both ELISA and PCR) in the category 50-249 mikling cows. All samples were positive in the other categories. In the Czech Republik only two samples were negative in the smallest herd size category (50-249). All samples were ELISA and/or PCR positive in the categories 250-499, 500-999 and ≥1000 mikling cows. In Slovenia and Serbia, where smaller dairy family farms dominated, ELISA and/or PCR positivity was 50% and 56.25% in the first category (50-249). Surprisingly, Croatian dairy farms showed 100% positivity in all herd categories, possibly due to the high density of dairy farms.

The number of examined dairy herds varied by countries, but overall *C. burnetii* infection status (percentage of positive herds/total number of herds with ELISA and PCR tests) ranged between 50-100.00% in the Central and Eastern European countries. The analysis of ELISA and PCR test results in association with herd sizes revealed that herds with ≥250 animals showed significantly higher *C. burnetii* positivity (positive test results: 100%; Spearman's rank correlation, rho = 0.716, $p < 0.001$), than herds with <250 animals (positive test results: 73.03%). On the other hand, when examining only PCR test results, similar percentages of positive milk samples (40.63-44.94%) were detected among the herds of different sizes. Results of the ELISA tests and PCR assays of the 370 bulk tank milk samples are summarized in Table 5.

Table 5. Summary of *C. burnetii* specific ELISA and PCR test results of bulk tank milk samples originating from Central and Eastern Europe.

Herd size[a]	Number of herds	ELISA[b]	PCR[c]	ELISA & PCR[d]	Infection status[e]	
50-249	4	2	0	2	4/4	100.00%
250-499	5	2	0	3	5/5	100.00%
500-999	4	2	0	2	4/4	100.00%
Total CRO	**13**	**6**	**0**	**7**	**13/13**	**100.00%**
50-249	30	10	1	17	28/30	93.33%
250-499	70	45	0	25	70/70	100.00%
500-999	34	19	0	15	34/34	100.00%
≥1000	4	2	0	2	4/4	100.00%
Total CZ	**138**	**76**	**1**	**59**	**136/138**	**98.55%**
50-249	7	2	0	2	4/7	57.14%
250-499	32	21	0	11	32/32	100.00%
500-999	66	40	0	26	66/66	100.00%
≥1000	21	13	0	8	21/21	100.00%
Total HU	**126**	**76**	**0**	**47**	**123/126**	**97.62%**
50-249	16	5	0	4	9/16	56.25%
250-499	5	3	0	2	5/5	100.00%
≥1000	3	1	0	2	3/3	100.00%
Total SRB	**24**	**9**	**0**	**8**	**17/24**	**70.83%**
50-249	20	5	0	10	15/20	75.00%
250-499	14	2	0	12	14/14	100.00%
500-999	15	5	2	8	15/15	100.00%
≥1000	4	3	0	1	4/4	100.00%
Total SK	**53**	**15**	**2**	**31**	**48/53**	**90.56%**
50-249	12	2	0	4	6/12	50.00%
250-499	4	0	0	4	4/4	100.00%
Total SLO	**16**	**2**	**0**	**8**	**10/16**	**62.50%**

[a]Ranges of number of animals in the examined herds are given according to country of origin.

[b]Number of positive results only with ELISA tests.

[c]Number of positive results only with PCR tests.

[d]Number of positive results both with ELISA and PCR tests.

[e]Total number and percentage of positive results with ELISA and PCR tests

5.2. Results of the ELISA tests of blood samples in different hosts species

ELISA testing showed individual seropositivity in 258 out of 547 (47.2%) cows examined and in 69 out of 271 (25.5%) small ruminants tested, among them in 47 out of 200 sheep (23.5%) and in 22 out of 71 goats (31.0%). *C. burnetii* antibodies were not found in zoo animals. Cattle were more likely to be seropositive than small ruminants ($p<0.0001$) and zoo animals ($p<0.0001$), as well as small ruminants compared to zoo animals ($p=0.0002$). After controlling for geographical region, cattle were 4.32 times more likely (95% confidence interval of odds ratio: 2.13–8.75, $p<0.0001$) to be seropositive compared to small ruminants. No significant difference at animal-level seropositivity was found between regions ($p=0.697$). Seropositivity was demonstrated in 44 out of 44 (100%) dairy cattle farms, with at least one serum sample found to be positive on each farm. The seropositivity rate of small ruminant farms was 55.0% (11 positive out of 20 tested), with 9 out of 16 (56.3%) sheep flocks and 2 out of 4 (50.0%) goat herds showing seropositivity. The ELISA test results on mixed host species and their origin are summarised in Table 6.

Table 6. Summary of Enzyme-linked immunosorbent assay (ELISA), results of the examined cattle herds and sheep and goat flocks at individual and farm levels.

Statistical Large Region	**Planning and Statistical Region**	**Tested herds**	**Positive herds %**	**Tested animals**	**Seropositive animals %**
Transdanubia	Western Transdanubia	6	6 (100%)	88	41 (46.5%)
	Central Transdanubia	7	7 (100%)	97	46 (47.4%)
	Southern Transdanubia	6	6 (100%)	76	38 (50.0%)
Great Plain and North	Northern Hungary	7	7 (100%)	80	30 (37.5%)
	Northern Great Plain	9	9 (100%)	107	56 (52.3%)
	Southern Great Plain	9	9 (100%)	99	47 (47.7%)
Total dairy cattle		44	44 (100%)	547	258 (47.2)
Statistical Large Region	**–**	**Tested flocks**	**Positive flocks%**	**Tested animals**	**Seropositive animals%**
Transdanubia	–	8	4 (50%)	106	33 (31.1%)
Great Plain and North	–	12	7 (58.3%)	165	36 (21.8%)
Total Small Ruminants	–	20	11 (55.0%)	271	69 (25.5%)

5.3. Results of the IgG Phase I and Phase II *C. burnetii* antibodies in human blood samples

IgG Phase I antibodies were detected in 53 out of the 70 (75.7%) serum samples tested. In 59 out of the 70 individuals tested (84.3%), anti-*C. burnetii* IgG Phase II was detected. Among the IgG I seropositive individuals, antibodies were shown in 8 out of 8 veterinarians (100%), in 12 out of 12 inseminators (100%), in 22 out of 26 animal caretakers (84.6%), in 8 out of 17 parlour workers (47%) and in 3 out of 7 herd managers (42.8%). IgG Phase II antibodies to *C. burnetii* were found in 8 out of 8 veterinarians (100%), in 12 out of 12 inseminators (100%), in 26 out of 26 animal caretakers (100%), in 8 out of 17 milking parlour workers (47%), and in 5 out of 7 herd managers (71.4%) (Table 7; Table S3). The titres of IgG antibodies showed a wide variation; with only a few very high values greater than 1:256 (Table 8). There was a correlation between occupation and seropositivity rate. Applying Spearman's rank correlation, we found a statistically significant correlation between the length of employment and the percentage of positivity ($r = 0$; $P < 0.001$) among the pooled groups. There was no correlation between age and seropositivity rate. Seropositivity rate was 37.5% in farm staff employed at dairy farms for less than 1 year, 83.3% in farm workers with a length of employment between 2 and 5 years, and 94.7% in staff employed at dairy farms for more than 5 years (Table 9). We found a confirmed case of acute Q fever in one veterinarian during the past few years. The percentage of IgG positivity was found to be higher in men (89.4%) than in women (61.5%). Considering IgG phase I, high-risk and medium-risk groups had significantly higher chances of being seropositive compared to the low-risk group ($p=0.0001$ and $p=0.0099$, respectively). No statistically significant difference was found between the high-risk and medium-risk groups in the occurrence of IgG Phase I seropositivity ($p=0.1213$). Regarding IgG Phase II, the occurrence of seropositivity was significantly higher in the high-risk and medium-risk groups compared to the low-risk group ($p=0.0005$ and $p=0.0002$, respectively). No significant difference was found between high-risk and medium-risk groups in IgG Phase II seropositivity ($p=1.0000$).

Both IgG Phase I and Phase II antibodies were detected in 24 out of 24 (100%) serum samples tested in farm veterinarians by IFA. (Table 10).

Table 7. *C. burnetii* seroprevalence among different occupational groups in dairy farms.

Occupation	No. Tested	Phase II positive	Phase I positive
Veterinarian	8	8	8
Inseminator	12	12	12
Herd manager	7	5	3
Parlour worker	17	8	8
Animal caretakers	26	26	22
Total	70	59	53
		84.2%	75.7%

Table 8. Titre distribution of antibodies against *C. burnetii* Phase I and Phase II antigens in 70 Q fever high-risk subjects in Hungary.

	Phase I antigen		Phase II antigen	
Titre	**n**	**%**	**N**	**%**
Negative	17	24	11	16
<16	12	17	18	25
32	10	14	3	4
64	10	14	8	11
128	10	14	13	19
256	6	10	10	15
512	3	4	4	6
1024	2	3	3	4
Totals	**70**	**100%**	**70**	**100%**

Table 9. *C. burnetii* seroprevalence in dairy farm workers with different length of employment.

Length of employment	Total worker	Positive	Negative
1 year	8	3 (37.5%)	5
2-5 year	24	20 (83.3%)	4
>5 year	38	36 (94.7%)	2

Table 10. Titre distribution of IgG antibodies of veterinarians (N=24) against *C. burnetii* Phase I and Phase II antigens in the context of length of employment in dairy farms.

NO.	Length of employment in dairy farm	Titre	Phase II	Titre	Phase I	Age	Sex
1	11	1:512	positive	1:1024	positive	58	Male
2	9	1:128	positive	1:64	positive	42	Male
3	8	1:128	positive	1:64	positive	48	Male
4	9	1:128	positive	1:64	positive	38	Male
5	15	1:128	positive	1:64	positive	42	Male
6	10	1:64	positive	1:64	positive	52	Male
7	3	1:256	positive	1:256	positive	32	Female
8	30	1:16	positive	1:32	positive	66	Male
9	7	1:64	positive	1:64	positive	33	Male
10	4	1:16	positive	1:16	positive	50	Male
11	42	1:64	positive	1:32	positive	68	Male
12	36	1:128	positive	1:64	positive	60	Male
13	10	1:16	positive	1:16	positive	51	Male
14	1	1:128	positive	1:128	positive	27	Male
15	3	1:16	positive	1:16	positive	31	Female
16	15	1:16	positive	1:16	positive	51	Male
17	8	1:16	positive	1:16	positive	41	Male
18	34	1:32	positive	1:32	positive	57	Male
19	8	1:64	positive	1:32	positive	33	Female
20	22	1:256	positive	1:128	positive	52	Male
21	26	1:1024	positive	1:512	positive	51	Male
22	16	1:32	positive	1:16	positive	43	Male
23	22	1:32	positive	1:32	positive	48	Male
24	16	1:256	positive	1:256	positive	41	Male

5.4. Results of *C. burnetii* seropositivity rate in cows that lost pregnancy in early stage

The avarege pregnancy rate was 61.9% (199/321) in the three tested dairy farms. The rate of pregnancies lost between days 29-35 and days 60-70 of the gestation period was found to be 18%. ELISA testing showed 52% individual seropositivity in the tested cows (Table S2). A higher percentage of *C. burnetii* positivity was noted in cows that had lost their pregnancy. The seropositivity of cows with pregnancy loss was 80.5%, while that of the pregnant animals was 48.2% (Figure 7). Statistical analysis showed a significant positive correlation between positive ELISA test results and the loss of pregnancy (Spearman's rank correlation, rho = 0.282, $p < 0.05$). ELISA positivity was greatly increased in cows which had lost pregnancy after the first breeding (94.4%), while in pregnant animals seropositivity was only slightly increased (53.8%) (Figure 8). Statistical analysis showed a significant positive correlation between positive ELISA test result and the loss of pregnancy at first AI (Spearman's rank correlation, rho = 0.446, $P < 0.05$).

In the dairy herds included in the study, an individual seropositivity rate of 66.6% was detected in previously ELISA-positive animals by CFT (Phase II), 38.8% of the cows exhibiting low titres (1:10–1:40) and 27.7% high (<1/80) titres. CFT (Phase I) detected 49.9% seropositivity in animals that had lost their pregnancy, with 41.6% of these cows exhibiting low titres (1:10–1:40) and 8,3% of them having high (<1/80) titres (Table 11). Statistical analysis showed a significant difference in CFT positivity between animals found pregnant in Phase I (37/96) and cows that had lost their pregnancy in Phase I (18/36) (Student's *t*-test, $P < 0.05$).

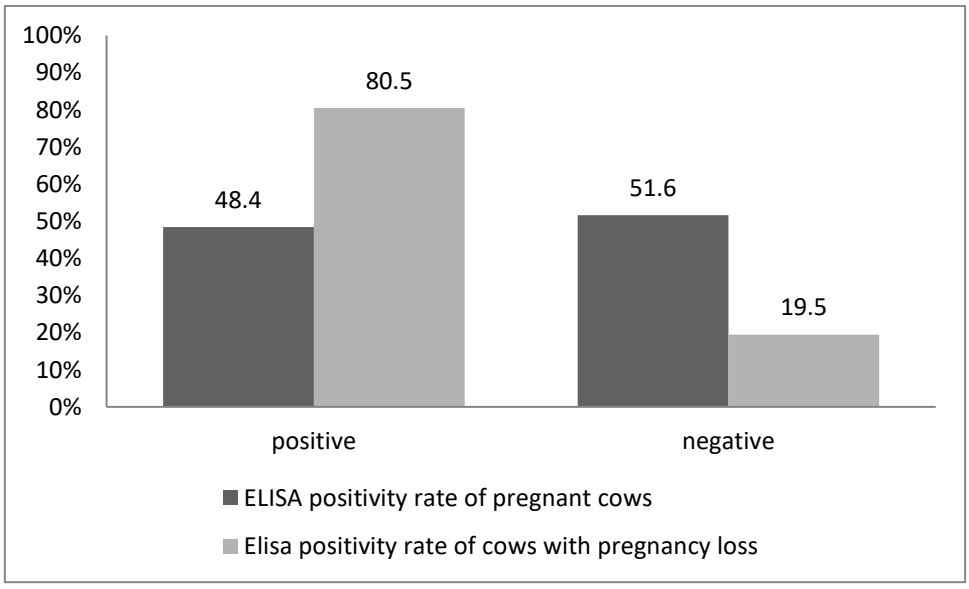

Figure 7. *C. burnetii* ELISA positivity and negativity rates of pregnant cows and of cows with pregnancy loss

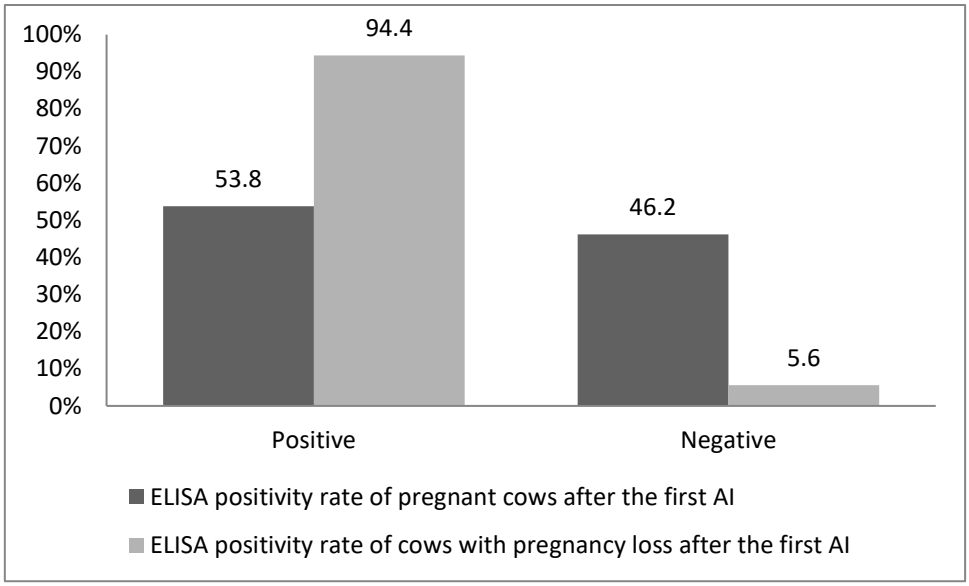

Figure 8. *C. burnetii* ELISA positivity and negativity rates in cows pregnant and in cows with pregnancy loss after the first artificial insemination

Table 11. Summary of complement fixation test (CFT) results of ELISA-positive pregnant cows and cows with pregnancy loss

	CFT Titers 1:10-1:40 latent infection	CFT Titers >1:80 Evolving Infection	CFT positive/Total ELISA positive
Pregnancy lost animals Phase II	14 (38.8%)	10 (27.7%)	24/36 (66.6%)
Pregnancy lost animals Phase I	15 (41.6%%)	3 (8.3%)	18/36 (50.0%)
Pregnant animals Phase II	40 (41.6%)	22 (23.0%)	62/96 (64.5%)
Pregnant animals Phase I	28 (29.1%)	9 (9.3%)	37/96 (38.5%)

5.5. Real-time PCR results of bovine cotyledons from retained and normally separated placentas

Eighty (88.9%) out of the 90 cotyledons from retained placentas and 31 (40.3%) out of the 77 cotyledons from normally separated placentas tested positive by IS*1111* real-time PCR (Table 12; Table S4). Seventeen (21.3%) out of these positive samples from retained placentas were highly loaded with *C. burnetii* with a Ct value less then 27.08, ranging between 11.92 and 27.08, while the rest of the positive samples were moderately loaded, with Ct values ranging between 28.43 and 36.91. High DNA load was not detected in normally separated placentas, in which we found only moderate DNA copy loads with Ct values ranging between 28.43 and 36.91. Among the 17 strongly positive samples from retained placentas, five out of the ten

samples giving the strongest positivity (4 Hungarian and 1 Slovakian, Ct 11.92–18.28) were genotyped by multispacer sequence typing (MST) based on ten loci, which revealed sequence type (ST) 61, a type that had not been detected in Hungary and Slovakia previously. They were deposited to GenBank and assigned to accession numbers MW441853–MW441902. (Figure 9). This sequence type differs from ST20 in locus Cox37, in which a deletion of a single nucleotide (T) at position 420 was noted compared to allele 4. After verification, the novel allele 37.10 for locus Cox37 was added to the online MST database (http://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/) and as a consequence the existence of the new sequence type named ST61 was confirmed. The allele profile of ST61 is 2-3-6-1-5-10-4-10-6-5 for intergenic spacers Cox2-Cox5-Cox18-Cox20-- Cox22-Cox37-Cox51-Cox56-Cox57-Cox61, respectively. Retained placenta was recorded in 42.0% (21/50), 13.0% (6/46), and 88.7% (63/71) of cows in Parity 1, 2, and 3+, respectively. Retained placenta was more likely to occur in *C. burnetii* PCR-positive cows compared to their PCR-negative counterparts (OR = 12.61, 95% CI: 2.47–64.38, $P = 0.0023$). Parity was also significantly related to the occurrence of retained placenta ($P < 0.0001$). Each pairwise comparison between parities was significant, with both Parity 1 ($P = 0.0062$) and Parity 3+ ($P < 0.001$) having higher odds of retained placenta than Parity 2, and Parity 3+ having higher odds than Parity 1 ($P = 0.0079$).

C. burnetii positivity was detected in 12 out of 21 (57,1%) (12/21) by IHC from retained placenta. Necrotic area and foamy trophoblast cells at the edge of the lesion of the cotyledon were detected in the *C. burnetii* infected cells by IHC. (Figure 10).

Table 12. Percentage of *C. burnetii* positivity with different DNA loads in retained and normally separated placentas

	High DNA load (Ct ≤ 27.08)	**Moderate DNA load (Ct > 27.08)**	**Negative**
Retained placenta (n=90)	17 (18.9%)	63 (70.0%)	10 (11.1%)
Normally separated placenta (n=77)	–	31 (40.3%)	46 (59.7%)

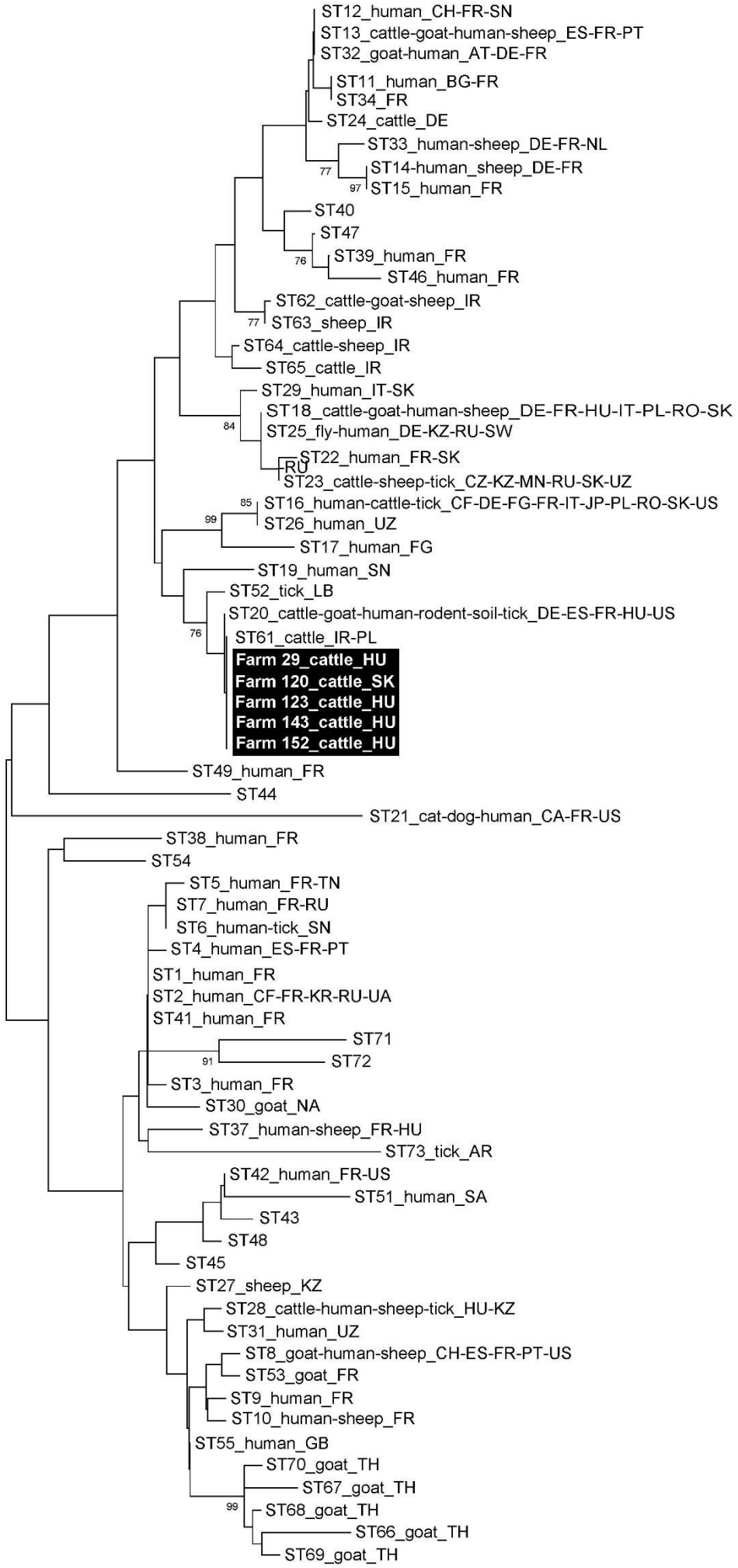
ST12_human_CH-FR-SN
ST13_cattle-goat-human-sheep_ES-FR-PT
ST32_goat-human_AT-DE-FR
ST11_human_BG-FR
ST34_FR
ST24_cattle_DE
ST33_human-sheep_DE-FR-NL
77
ST14-human_sheep_DE-FR
97
ST15_human_FR
ST40
ST47
76
ST39_human_FR
ST46_human_FR
ST62_cattle-goat-sheep_IR
77
ST63_sheep_IR
ST64_cattle-sheep_IR
ST65_cattle_IR
ST29_human_IT-SK
ST18_cattle-goat-human-sheep_DE-FR-HU-IT-PL-RO-SK
84
ST25_fly-human_DE-KZ-RU-SW
ST22_human_FR-SK
RU
ST23_cattle-sheep-tick_CZ-KZ-MN-RU-SK-UZ
85
ST16_human-cattle-tick_CF-DE-FG-FR-IT-JP-PL-RO-SK-US
99
ST26_human_UZ
ST17_human_FG
ST19_human_SN
ST52_tick_LB
ST20_cattle-goat-human-rodent-soil-tick_DE-ES-FR-HU-US
76
ST61_cattle_IR-PL
Farm 29_cattle_HU
Farm 120_cattle_SK
Farm 123_cattle_HU
Farm 143_cattle_HU
Farm 152_cattle_HU
ST49_human_FR
ST44
ST21_cat-dog-human_CA-FR-US
ST38_human_FR
ST54
ST5_human_FR-TN
ST7_human_FR-RU
ST6_human-tick_SN
ST4_human_ES-FR-PT
ST1_human_FR
ST2_human_CF-FR-KR-RU-UA
ST41_human_FR
ST71
91
ST72
ST3_human_FR
ST30_goat_NA
ST37_human-sheep_FR-HU
ST73_tick_AR
ST42_human_FR-US
ST51_human_SA
ST43
ST48
ST45
ST27_sheep_KZ
ST28_cattle-human-sheep-tick_HU-KZ
ST31_human_UZ
ST8_goat-human-sheep_CH-ES-FR-PT-US
ST53_goat_FR
ST9_human_FR
ST10_human-sheep_FR
ST55_human_GB
ST70_goat_TH
ST67_goat_TH
99
ST68_goat_TH
ST66_goat_TH
ST69_goat_TH

Figure 9. Neighbour-joining tree showing the placement of the samples (highlighted area) from this study with known STs. Bootstrap values of ≥70 is shown (1,000 replicates). The scale bar represents the average number of substitutions per site. Isolate origins and sources are given according to the MST database using the following location codes: Argentina (AR), Austria (AT), Belgium (BG), Canada (CA), Central African Republic (CF), Czech Republic (CZ), Ethiopia (ET), France (FR), French Guiana (GF), Germany (DE), Greece (GR), Hungary (HU), Italy (IT), Iran (IR), Japan (JP), Kazakhstan (KZ), Kyrgyzstan (KR), Lebanon (LB), Mongolia (MN), Namibia (NA), Netherlands (NL), Poland (PL), Portugal (PT), Romania (RO), Russian Federation (RU), Saudi Arabia (SA), Senegal (SN), Slovakia (SK), Spain (ES), Sweden (SW), Switzerland (CH), Thailand (TH), Tunisia (TN), Ukraine (UA), United Kingdom (GB), United States (US), and Uzbekistan (UZ).

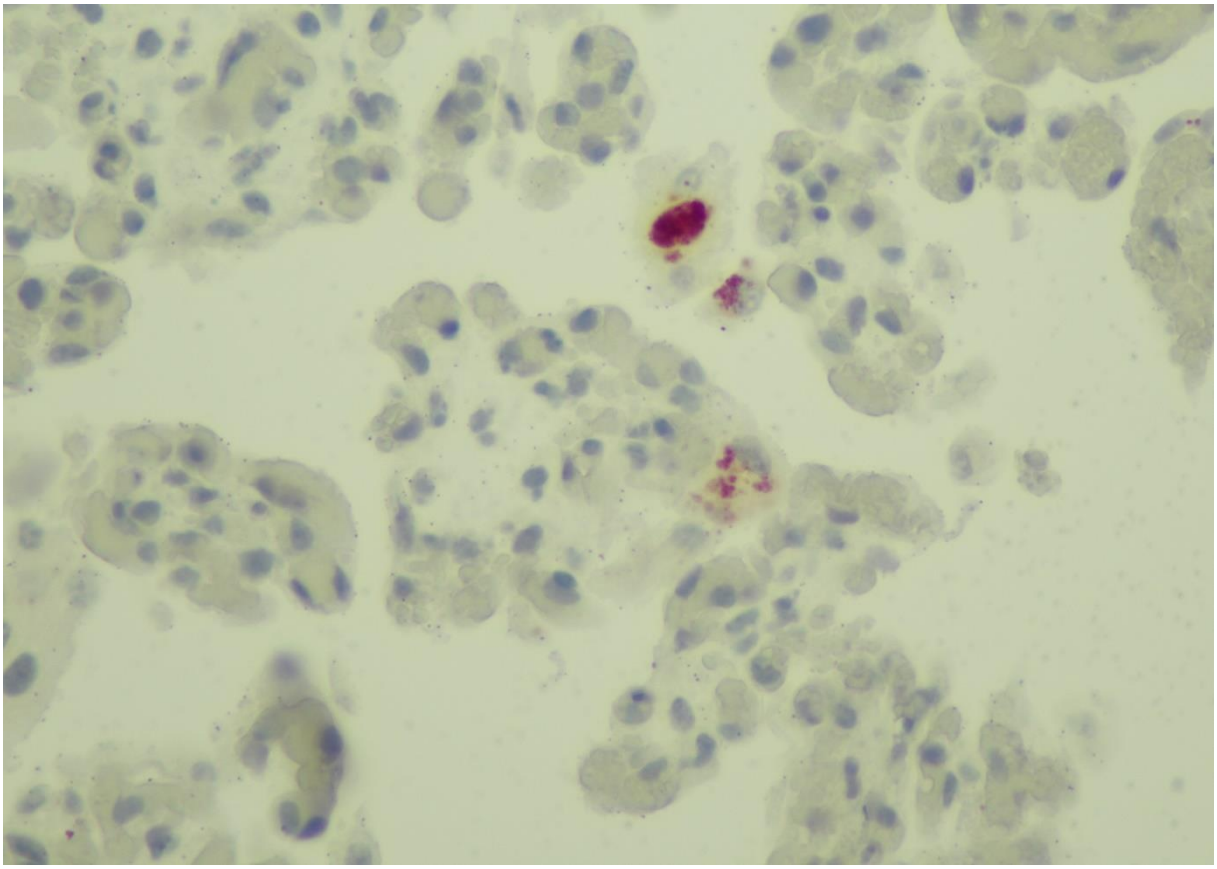

Figure 10. Cattle cotyledon section. *C. burnetii* in large cytoplasmic inclusions in the trophoblast cells, and less frequently on the surface of the layer of trophoblast cells. Labelled streptavidin-biotin method, counterstaining with Mayer's haematoxylin. × 200

6. Discussion

6.1. Prevalence of *C. burnetii* in Central and Eastern European dairy herds

Detection of the pathogen in the examined Central and Eastern European region varied according to country of origin with positivity ranging between 33.33-43.48% (Czech Republic, Hungary, Serbia) and 50.00-62.26% (Croatia, Slovakia, Slovenia). Similarly, previous publications in Europe showed 24-40.1% (Belgium, Denmark, France, Italy, Poland, Portugal, Switzerland) and 51.7-69.7% (England, Germany, Spain) prevalence of the pathogen in bulk tank milk samples (Czaplicki et al., 2012; Angen et al., 2011; Guatteo et al., 2012; Valergakis et al., 2012; Baumgartner et al., 2011; Valla et al., 2014; Astobiza et al., 2011; Astobiza et al., 2012; Anastacio et al., 2016; Hilbert et al., 2015). In the present study, detection of *C. burnetii* specific antibodies showed higher positivity than the PCR assays in all countries, ranging between 62.50-70.83% (Serbia, Slovenia) and 86.79-100.00% (Croatia, Czech Republic, Hungary, Slovakia). Previous publications reported lower ELISA positivity of bulk tank milk samples from Europe, ranging between 25-37.9% (Greece, Ireland, Portugal) and 45.5-78.6% (Belgium, Denmark, the Netherlands, Poland, Spain) (Czaplicki et al., 2012; Astobiza et al., 2011; Anastacio et al., 2016; Hilbert et al., 2015; Agger et al., 2010; Ryan et al., 2011).

Positive correlation was found between herd size and percentage of *C. burnetii* positive results (with PCR and/or ELISA tests), assuming that herds of 250 animals or more (with farm structures resulting in crowded herds) are at higher risk for *Coxiella* infection. The observed negative influence of increasing herd size and cattle density on the risk of *C. burnetii* detection was described before by analyzing coxiellosis in Danish dairy herds (Agger et al., 2014). The average herd size is the highest in the examined region among the European countries, with average number of animals/herds being 217 in Slovakia, 138 in the Czech Republic and 76 in Hungary. Moreover, there are 32 industrial dairy herds in Hungary, which hold more than 1000 dairy cows, and the concentration of Hungarian dairy population is the highest in Europe (Comission Eu dairy farms report, 2013). Average cattle herd sizes in Serbia and Slovenia are between 12-19 animals; and accordingly, the detected prevalence of *C. burnetii* in these countries was lower (positive ELISA and/or PCR results 70.83% and 62.50%, respectively) than in the other examined countries.

The present research assessed the prevalence of Q fever at dairy farms in Central and Eastern European countries, revealing increased seroprevalence in bulk tank milk samples compared to other European countries. Based on the analysis of the data, it is assumed that with growing numbers of animals in dairies and farm structures moving toward concentration, the risk of *C. burnetii* prevalence is increasing, underlining the importance of monitoring the herds' infection status and implementation of control measures.

6.2. *C. burnetii* infection in dairy cattle, sheep, goats and zoo animals in Hungary

This research found different *C. burnetii* infection rates in the different animal species tested. Most seroepidemiological studies indicate that the seroprevalence of antibodies to *C. burnetii* is higher in cattle than it was 20–30 years ago (Maurin and Rault, 1999). The present study found 47.2% seropositivity in cattle, which is higher than that reported previously (38%) in Hungary (Gyuranecz et al., 2012). In a recent study we found 52% *C. burnetii* seropositivity, but this only focused on early pregnancy loss in three Hungarian dairy farms and was not as large-scale and representative as the present research. Compared with the European average (20%), the seroprevalence found in cattle is much higher (Guatteo et al., 2011). According to a recent survey, seroprevalence among sheep in Hungary was 6% by ELISA (Gyuranecz et al., 2012). The present study found 23.5% seropositivity in sheep, which is also higher (15%) than the European average (Guatteo et al., 2011). However, *C. burnetii* seropositivity at the individual level in sheep shows huge differences among countries. Animal-level seroprevalence was 1.8% for sheep in Sweden (Magouras et al., 2017) and 16.3% in Italy (Rizzo et al., 2016). Sheep-level seroprevalence was found to be 14.7% in Canada, and it was higher in dairy sheep (24.3%) than in meat sheep (10.2%) (Meadows et al., 2015). Hungary has a relatively small national goat flock (54,000 goats; http://mjksz.hu), which is usually kept in flocks of 1–50 animals per farm. No previous serological survey on *C. burnetii* infection was available regarding Hungarian goat farms. Only a single caprine *C. burnetii* abortion case was diagnosed and reported in 2006 (Szeredi et al., 2006). In this study, the four biggest Hungarian goat farms (flock size: 300–500 animals) were tested and found to have 31.0% seropositivity by ELISA. There is a correlation between the incidence of Q fever and goat density. In the Netherlands there was a 75-fold increase in the goat population between 1985 and 2009, and the country faced one of the largest Q fever outbreaks in the world (Eldin et al., 2016). According to a large-scale study conducted in the Netherlands in 2008, 21.4% of the goats were seropositive for antibodies to *C. burnetii*, while farm prevalence was 43.1% (Schimmer et al., 2011). However, wildlife can also constitute a reservoir and *C. burnetii* infection was confirmed in some zoos (Clemente et al., 2008). We could not find seropositive animals among different species at the three biggest zoos in Hungary. In Africa, some animal species such as camels are significant reservoirs of the disease. Schelling et al. (2003) reported 80% *C. burnetii* seropositivity among camels in Chad, Bellabidi et al. (2020) found 75.5% seroprevalence of *C. burnetii* antibodies in Algeria, but *C. burnetii*-specific antibodies were detected in 40.7% of camels in Egypt as well (Klemmer et al., 2018). The present study has demonstrated the importance of Q fever, which is widespread in dairy cattle, but sheep and goats also appear to pose a major risk as sources of human infection in Hungary.

6.3. *C. burnetii* infection rate among dairy farm workers and veterinarians

The number of annually reported acute human Q fever infections in Hungary ranged between 28 and 48 from 2015 to 2019 (ECDC report). Although some of these cases were associated with farm workers (Balla, unpublished data), unfortunately there are no official reports about them. A recent study has found that *C. burnetii* infection rapidly increased in Hungarian dairy farms due to the growing number of animals in dairy units and farm structures moving towards concentration. The prevalence of *C. burnetii* was found to be 97.6% based on ELISA and PCR test findings in bulk tank milk. Samples of retained placenta from Hungarian dairy herds showed 65.2% *C. burnetii* positivity by PCR and 57.1% positivity by immunohistochemistry (IHC). A larger herd size could pose a risk because of the increased chance of *C. burnetii* introduction or the presence of a larger susceptible population of cows. Cattle shed *C. burnetii* mainly with birth products such as the placenta, amniotic fluids, and vaginal mucus (Guatteo et al., 2006). These bacteria are also shed in the milk, faeces and urine, but the most important sources of *Coxiella* transmission from animals to humans are birth products (Guatteo et al., 2006). As the surveyed large dairy farms have about 600 to 1,000 calvings per year, the chance of farm workers to acquire *Coxiella* infection is much higher than in smaller family farms. Many studies have reported that *C. burnetii* was found in parturient bovine placentae (Hansen et al., 2011; Botta et al., 2019). However, *C. burnetii* is generally inactive in the fetal membranes, and human Q fever outbreaks are rarely associated with cattle; but infected placental membranes provide a huge opportunity for the dissemination of viable organisms to the environment to infect both humans and cattle (Hansen et al., 2011; Luoto et al.,1950). The present study has demonstrated a high prevalence of *C. burnetii* antibodies in dairy farm workers. All workers had daily contact with dairy cattle and although we found different seropositivity rates among different occupational groups, it is difficult to compare our serological results with other findings from different countries, because the pooled groups were not entirely the same and they have used different screening tests and cut-off values. However, the 84.2% prevalence of IgG Phase II and the 75.7% prevalence of IgG Phase I antibodies to *C. burnetii* found in Hungarian farm workers were much higher than the seropositivity rates demonstrated in English farmers (27%) (Thomas et al., 1995) or in Polish farmers (17.8%) (Cisak et al., 2003). A recent study has found 24.1% seropositivity among Ecuadorian farm workers (Echeverria et al., 2019). A Southern Italian survey conducted in Sicily found 21.4% and 25% prevalence of anti-*Coxiella* antibodies in female and male farm workers, respectively (Fenga et al., 2015). Very low prevalence of *C. burnetii* IgM immunoglobulin (4.6%) was detected in livestock workers in Trinidad (Adesiyun et al., 2011). A large-scale study detected an 11% seroprevalence rate of anti-*C. burnetii* IgG among humans coming into contact with dairy cattle in Denmark, but only 3% of the surveyed Danish dairy farmers were seropositive

(Bosnjak et al., 2010). The seroprevalence rate of dairy cattle farm residents was the highest (72.1%) in the Netherlands of all values reported all over the world (Schimmer et al., 2014). In the current study, we found 100% seropositivity among veterinarians and inseminators. We detected the highest prevalence in veterinarians which is consistent with research findings from many other countries. In Denmark 36% of the veterinarians tested had antibodies while only 2% of inseminators were seropositive (Bosnjak et al., 2010). Among veterinarians, the seroprevalence of antibodies to *C. burnetii* was 13.5% in Japan (Abe et al.,2001), 9.5% in Austria (Nowotny et al., 1997) and 22.2% in the USA (Whitney et al., 2009). According to an Estonian study, the prevalence of *C. burnetii* antibodies was 9.62% among veterinary professionals, but among farm animal veterinarians the seroprevalence was significantly higher, 17.39% (Neare et al., 2019). The prevalence of *C. burnetii* seropositivity found in Dutch livestock veterinarians was 69.2% (Wielders et al., 2015). Many international studies clearly indicate that farm veterinarians are the most important occupational risk group (Wielders et al., 2015). The reason why Hungarian farm veterinarians had such a high *C. burnetii* infection rate was probably their intensive contact with highly infected dairy farms. Veterinarians work with sick animals during parturition, remove retained fetal membranes, treat metritis, flush out the uterus, and they are exposed to contact with infected placenta and birth products on a daily basis. Inseminators of large industrial dairy herds often have the same duties as veterinarians, and therefore they constitute the second most important occupational risk group. We found 100% seropositivity among animal caretakers, who are also at a high risk of becoming infected with *C. burnetii* because of their close contact with infected cattle. They also have a close contact with bedding materials, which are also a source of *Coxiella* transmission from animals to humans (Guatteo et al., 2006). We found 47% seropositivity among milking parlour workers and 71.4% among herd managers. These two occupational groups had less contact with animals and mostly with birth products. In milk, sporadic shedding of *Coxiella* is the most common kinetic pattern (Guatteo et al., 2007). As the milking machine is a relatively closed system, milk is probably not a common source of *Coxiella* transmission from cattle to milking parlour workers. Herd managers are regularly present on dairy farms to manage the milk production, but sometimes they assist with calvings or act as substitutes for inseminators; in such cases they have a higher chance of becoming infected with C. *burnetii*. In 2 out of the 70 subjects (2.8%) examined in this study we found significantly elevated titres of IgG Phase I antibodies to *C. burnetii* equal or greater than 1:1024 titres indicating the likelihood of chronic Q fever and warranting further clinical examinations (Dupont et al.,1994). This present study has demonstrated that IgG Phase I and Phase II antibodies to *C. burnetii* are higher in Hungarian dairy farm workers than those described in several international seroepidemiological studies among different occupational groups in other European countries. Veterinarians are the occupational group most exposed to infection, but inseminators and

animal caretakers are at a similarly high risk of infection in industrial dairy farms. The high *Coxiella* burden in dairy farms underlines the importance of controlling the development of chronic Q fever among occupationally exposed people, such as dairy farm workers as well as the need for implementation of some preventive measurements. The prevalence of *C. burnetii* was found to be very high among dairy cattle farms in our recent study and we found high human seroprevalence of *C. burnetii* among dairy farm workers as well, in line with what we had previously assumed. Our study also has demonstrated that high *C. burnetii* seroprevalence among dairy farm workers correleted with the high prevalence of *C. burnetii* in Hungarian dairy herds.

6.4. Bovine Coxiellosis in the context of early pregnancy loss in dairy cows

Several studies have investigated early pregnancy loss in cows between days 28 and 98 after AI. In intensively managed dairy farms of North America the rate of late embryonic loss was found to be 20.2% (Vasconselos et al., 1997). Silke et al. (2001) reported 7.2% late embryonic loss during the same period for dairy cows kept mainly in pasture-based milk production systems in Ireland. López-Gatius (2003) described 10.2% pregnancy loss from gestation day 38 to 90 in lactating dairy cows from a single herd in Northern Spain. Zobel et al. (2011) reported a pregnancy loss rate of 7.79% (in cows and heifers) on two Simmental dairy farms in Croatia from day 32 to 86 of gestation. In Hungary, a large number of dairy cattle were tested for pregnancy by assaying serum PSPB concentration at 29–35 days after insemination, and pregnancy was checked again by transrectal palpation 60–70 days after AI. A pregnancy loss of 19.3% was detected by assaying more than 10.000 blood samples (Gábor et al., 2007). The present study found 18.0% pregnancy loss, which is higher than previously reported from several other countries. Some authors have stated that embryonic and fetal mortality was not related to the genetic merit of cows (Diskin and Morris, 2008). No significant effect of previous synchronisation on the rate of pregnancy loss was found (López-Gatius et al., 2002). There is evidence that body condition may affect the pregnancy loss rate. Change of body condition was found to increase the incidence of embryonic mortality between days 28 and 56 of gestation (Silke et al., 2001). Negative energy balance during early gestation reduces fertility and may increase pregnancy loss. López-Gatius et al. (2002) found an about 2.4 times higher risk of pregnancy loss in cows that lost one unit in body condition compared to cows maintaining their body condition. Most authors have found a significant correlation between the incidence of embryonic loss and cow parity (Nyman et al., 2018). Some authors have reported an increase in late embryonic loss with increasing parity (Balendran et al., 2008) and with cow age and endocrine causes (Lee and Kim, 2007; Bajaj and Sharma, 2011). The risk

of pregnancy loss was found to be 3.1 times higher in cows with twin pregnancy, as reported by López-Gatius et al. (2012). The uterine environment and periparturient diseases such as subclinical endometritis has also been linked with pregnancy loss (Santos et al., 2004). A recent study has found an association between *C. burnetii* infection and endometritis, which may also be related to progressive reproductive disorders such as infertility (De Biase et al., 2018).

Infectious agents may also be associated with embryonic and fetal loss (Vanroose et al., 2000). Some viruses such as bovine viral diarrhoea virus (BVDV), bovine herpesvirus-1 (BoHV-1) and Bluetongue virus can cause pregnancy loss. BVDV is able to reach the embryo and infect it before the placenta is completely formed at around 30–32 days of pregnancy, resulting in embryonic death (McGowan and Kirkland, 1995; Tsuboi et al., 2011). BoHV-1 may be associated with decreased fertility and abortion in early to late gestation. The virus induces the development of chronic necrotising endometritis 31–47 days after artificial insemination (Graham, 2013). Infection of cattle by Bluetongue virus in early stages of pregnancy can result in early fetal death, but this virus infection is closely linked with late abortion and some serious malformations (Sperlova and Zendulkova, 2011). Bacterial, protozoal or fungal infections may cause early fetal death but are more closely associated with abortion. The protozoan pathogen *Neospora caninum* is a well-studied abortifacient infectious agent in cattle. Several publications state that *N. caninum* is the leading infectious cause of bovine abortions but is not associated with early pregnancy loss (Wilson et al., 2016).

The individual seroprevalence rates of *C. burnetii* in the dairy cows tested in this study (52%) were above both the international average (20.0%; Guatteo et al., 2011) and previous Hungarian findings (38%; Gyuranecz et al., 2012). The *C. burnetii* seroprevalence rate was much higher in animals that had lost their pregnancy (80.5%) than the rate found in pregnant cows or the average individual value. Seroprevalence rate was close to 100% in first-inseminated heifers that lost their pregnancy (94.4%).

An average individual seropositivity rate of 50% was detected by CFT (Phase I) in animals that had lost their pregnancy. Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above indicate an active phase of infection. A CFT titre of 1:40 is diagnostic for acute Q fever (Fournier et al., 1998). According to these CFT results both acute and chronic Q fever can occur during pregnancy. We detected a significantly higher percentage of phase I titres by CFT in animals that had lost their pregnancy. This means that these animals were in the chronic phase of the disease. In mammals, *C. burnetii* can be reactivated during pregnancy and thus cause reproductive problems (Fournier et al., 1998). Infection with *C. burnetii* at an early stage of gestation increases the chance of pregnancy loss.

The findings of this study indicate an association between pregnancy loss of dairy cows at the early stage of gestation and *C. burnetii* infection. The high prevalence of *C. burnetii* in dairy farms is a possible major risk factor related to pregnancy loss.

6.5. Prevalence of *C. burnetii* in bovine placentas in Hungary and Slovakia; detection of a novel sequence type

The high prevalence of *C. burnetii* on dairy farms may be a risk factor for human infection and it is also related to *C. burnetii*-associated reproductive disorders such as abortion, premature delivery, stillbirth, and weak offspring complex (APSW complex), early pregnancy loss and the retention of fetal membranes (Agerholm, 2013; Rahal et al., 2018). A similar large-scale study found a 52.9% rate of *C. burnetii* positive cases among 170 cotyledons from dairy cattle by real-time PCR targeting the *IS1111a* and *icd* genes in Denmark (Hansen et al., 2011). In that study involving 19 herds, the farm owners also selected and sampled one cotyledon per fetal membrane, but they did not record whether the cotyledon had originated from a normally separated or a retained placenta. Compared to that study, our research has found a higher rate of placental infection with *C. burnetii* in the retained fetal membranes (88.9%) and a similar infection rate in normally separated placentas (40.3%). Rahal et al. (2018) found 19.1% positivity among the placentas tested by real-time PCR targeting the *IS1111* gene in Algeria. Those samples were mainly collected from aborted cows, and only four placental samples originated from cows with normal delivery. That study found only two out of 14 samples (14.3%) highly loaded with *C. burnetii* (Ct values ranging between 16.2 and 21.2). We found 17 cotyledons highly loaded with *C. burnetii* (Ct values ranging between 11.92 and 27.08) among 111 positive samples (15.3%), which shows a similar percentage to that of samples from aborted cows. Some studies also found that the placentas of many parturient cows were infected by *C. burnetii* (Luoto et al., 1950), and 7.3% *C. burnetii* positivity was found by PCR in bovine cotyledons in the United Kingdom (Pritchard et al., 2011). We detected large amounts

of bacteria in retained fetal membranes and found a strong statistical association between the presence of *Coxiella* organisms and the occurrence of retained fetal membranes in dairy cows. A recent well-designed study has found that placental inflammation is more common in cases with lower Ct values, which means a higher bacterial load (Botta et al., 2019). Although *C. burnetii* rarely causes abortion in cattle, some studies have found an association between placentitis in cattle and the presence of these bacteria (Bildfell et al., 2000, as determined by the immunohistochemical staining of fixed placenta samples (Botta et al., 2019). Hansen et al. (2011) demonstrated that *C. burnetii* infection of the placenta causes mild cotyledonary changes which may explain why bovine Q fever is mostly subclinical. Pregnant cattle have 75–125 placentomes, and most authors including us examined only one cotyledon per membrane. Thus, we do not have appropriate information about all placentomes of pregnant cows. Although the possible role of *C. burnetii* infection during gestation in cattle is not fully clarified, *Coxiella*-infected placental tissue obviously acts as a possible source of human Q fever. MST20 is the predominant genotype worldwide among cattle; however, other genotypes have also been identified in the bovine species (Eldin et al., 2017). A recent study has also confirmed that *C. burnetii* (MST) sequence type ST20 is circulating on dairy farms in Algeria (Rahal et al., 2018). Previously the ST20 genotype had also been identified in cattle in Hungary (Sulyok et al., 2014). Strains belonging to the ST23 group have been reported in ticks and humans in Slovakia (Di Domenico et al., 2018), but this is the first description of ST61 in cattle in Hungary and Slovakia. This sequence type has been recently described from cattle in Brazil, Argentina, and Poland (Mioni et al., 2019; Szymańska-Czerwińska et al., 2019). The MST profile of the samples was ST61, which is the sequence type most often associated with bovine samples and products globally (Santos et al., 2012; Tilburg et al., 2012; Olivas et al., 2016; Eldin et al., 2017).

The results of the present study indicate that the prevalence and the DNA load of *C. burnetii* are significantly higher in retained fetal membranes than in normally separated placentas, and this may act as a possible risk factor for human infection mostly in workers and veterinarians treating cows with retained placentas.

The new sequence type ST61 and the ST20 genotype previously found in Hungary are still the primary causes of bovine coxiellosis in the region. Monitoring the herds' infection status and implementing biosafety control measures such as systematically collecting and destroying placenta and aborted fetuses can be adopted in dairy farms in order to prevent the disease, to reduce the spread of the pathogens, and to reduce environmental contamination and human infections.

7. Overview of the new scientific results

Ad 1. The prevalence of Q fever at dairy farms in Central and Eastern European countries, revealing increased seroprevalence in bulk tank milk samples compared to other European countries. *C. burnetii* specific ELISA showed 100.00% positivity in all examined countries if herds consisted of 250 milking cows or more. The growing number of farms managing large number of animals, where cattle density is high correlates with the increasing prevalence of *C. burnetii* in the region.

Ad 2. *C. burnetii* is mostly widespread in dairy cattle, but sheep and goats also appear to pose a major risk among the different host species in Hungary. Our large-scale study demonstrated the importance of Q fever in different species as a possible source for human infection in most regions of Hungary.

Ad 3. Our study found that veterinarians, inseminators, and animal caretakers had 100% seropositivity rate of antibodies Phase II. of *C. burnetii* in Hungarian dairy farms. All occupational groups in dairies are highly exposed to *C. burnetii* infection. Our study has also demonstrated that high *C. burnetii* seroprevalence among dairy farm workers correleted with the high prevalence of *C. burnetii* in Hungarian dairy herds.

Ad 4. The study found a higher *C. burnetii* seropositivity rate in cows that had lost their pregnancy than cows which were pregnant. Seropositivity rate was found to be much higher in first-bred cows that had lost their pregnancy at an early stage. The high prevalence of *C. burnetii* in dairy farms might potentially contribute to an increased risk of pregnancy loss.

Ad 5. The results of the present study indicate that prevalence and DNA load of *C. burnetii* in retained fetal membranes is significantly higher than in normally separeted placentas and this may act as a potentially higher risk factor for human infection mostly in workers and veterinarians treating cows with retained placentas.

Ad 6. Retained placentas genotyped by multispacer sequence typing (MST) based on ten loci, revealed sequence type (ST) 61, which had not been found previously in Hungary and Slovakia. The new sequence type ST61 and the ST20 genotype previously found in Hungary are still the primary causes of bovine coxiellosis in the region.

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9. Scientific publications

Publications on the topic of the thesis:

In peer-reviewed journals

Dobos, A., Kreizinger, Z., Kovács, A., Gyuranecz, M.: **Prevalence of *Coxiella burnetii* in Central and Eastern European dairy herds,** Comparative Immunology Microbiology & Infectious Disease, 72, 101489, 2020.

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Dobos, A., Fodor, I.: **Prevalence of *Coxiella burnetii* in bovine placentas in Hungary and Slovakia; detection of a novel sequence type,** Acta Veterinaria Hungarica, 69, 2021 online

Dobos, A., Fodor,I., Tekin,T., Đuričić, D., Samardzija, M., **: Presence of *Coxiella burnetii* in dairy cattle and farms in the Czech Republic**, Polish Journal of veterinary sciences [accepted] 2022

Conference oral presentations

Dobos, A., Kreizinger, Zs., Kovács, Á., Gyuranecz, M.: **Tejelő szarvasmarha állományok *Coxiella burnetii* fertőzöttségének vizsgálata közép-kelet európai országokban,** Akadémiai beszámolók, Budapest, 2020.

Dobos, A., Fodor, I., Kiss, G., Gyuranecz, M**.: Coxiella burnetii fertőzöttség szerológiai felmérése tejelő szarvasmarha, juh, kecske és állatkerti állatokban Magyarországon**, Akadémiai beszámolók, Budapest, 2022

Dobos, A., Kreizinger, Zs., B. Kovács, Á., Gyuranecz, M. **: Prevalence of *Coxiella burnetii* in Central and Eastern European dairy herds,** Buiatric Congress, 2021, Brno, Czech Republik, 2021.

Other Publications

in peer reviewed journals

Battay, M., Dobos, A., Illés Cs., Ózsvári L. : **Az afrikai sertéspestis gazdasági hatásai Észak-Kelet Pest és Nógrád megye vadgazdálkodására, különös tekintettel a klasszikus sertéspestissel kapcsolatos korábbi tapasztalatokra,** Magyar Allatorvosok Lapja, 141, 39-46 , 2019.

Dobos, A., Fodor, I., Kreizinger, Z., Makrai, L., Dénes,B., Kiss,I., Đuričić,D., Kovačić, M., Szeredi.: **Infertility in dairy cows – Possible bacterial and viral causes,** Veterinarska stanica, 53, 2021

Đuričić, D., Dobos, A. , Grbavac, J., Stiles, C. , Bacan, I., Vidas, Željko, Marković, F., Kočila, P., & Samardžija, M.: **Climate impacts on reproductive performance of Romanov sheep in the moderate climate,** Journal of Animal Behaviour and Biometeorology, 10, 2021.

10. Supplements

Table S1. Background information and *C. burnetii* specific ELISA and PCR test results of the studied 370 bulk tank milk samples from Central and Eastern Europe.

Farm /sample ID	Month of collection	**Country of origin**	**Town of origin**	**Herd size**	**Results of real-time PCR**	**Results of real-time PCR (Ct values)**	**ELISA results[a]**	**ELISA S/P %**	**Dilution of sample for ELISA testing**
1	October	Croatia	Osijek	50	positive	37.01	positive	102.86	1:50
2	October	Croatia	Osijek	90	negative	-	positive	132.47	1:50
3	October	Croatia	Gorjani	130	negative	-	positive	3,96 / 161,63	1:50/cc
4	October	Croatia	Dakovo	160	positive	30.11	positive	75.03	1:50
5	October	Croatia	Grube	250	negative	-	positive	51.57	1:50
6	October	Croatia	Vrpolje	280	positive	36.75	positive	60.16	1:50
7	October	Croatia	Kapelna	300	positive	32.92	positive	99.18	1:50
8	October	Croatia	Vlskovci P	300	positive	34.94	positive	145.57	1:50
9	October	Croatia	Satnica	300	negative	-	positive	74.62	1:50
10	October	Croatia	Diakovar	500	negative	-	positive	75.99	1:50
11	October	Croatia	Kucanci	500	positive	34.33	positive	83.08	1:50
12	October	Croatia	Diakovar	550	negative	-	positive	75.03	1:50
13	October	Croatia	Diakovar	700	positive	36.08	positive	88.54	1:50
14	April	Czech Republic	Písek	50	negative	-	negative	6.72	cc
15	July	Czech Republic	Libor	50	negative	-	positive	176.10	cc
16	June	Czech Republic	Kojetin	60	negative	-	negative	264.30	cc
17	March	Czech Republic	Velky Bor	75	positive	29.30	negative	11.54	cc
18	March	Czech Republic	Dacice	100	negative	-	positive	254.13	cc
19	March	Czech Republic	Malec	150	positive	33.50	positive	218.95	cc
20	March	Czech Republic	Loket	150	positive	36.20	positive	209.45	cc
21	April	Czech Republic	Dobsice	150	positive	36.90	positive	149.37	cc
22	May	Czech Republic	Chotebor	150	negative	-	positive	394.40	cc
23	June	Czech Republic	Luka	150	positive	38.10	positive	249.89	cc
24	June	Czech Republic	Nachod	150	positive	35.30	positive	237.70	cc
25	April	Czech Republic	Dobesice	160	positive	31.50	positive	180.57	cc
26	April	Czech Republic	Cicenice	160	positive	32.90	positive	138.23	cc
27	April	Czech Republic	Vodnany	160	negative	-	positive	129.20	cc
28	May	Czech Republic	Paseky nad Jizerou	160	positive	32.10	positive	478.10	cc
29	July	Czech Republic	Tresne	160	positive	36.00	positive	221.20	cc
30	July	Czech Republic	Bilsko	160	positive	35.60	positive	409.80	cc
31	April	Czech Republic	Pojbuky	180	negative	-	positive	155.97	cc
32	March	Czech Republic	Prestovice	200	negative	-	positive	284.06	cc
33	May	Czech Republic	Tremosna	200	positive	37.10	positive	435.80	cc
34	May	Czech Republic	Jihlava	200	positive	34.50	positive	433.80	cc
35	June	Czech Republic	Dublovice	220	positive	34.30	positive	453.10	cc

36	June	Czech Republic	Smetanova lhota	220	negative	-	positive	411.40	cc
37	August	Czech Republic	Kralovice	240	positive	36.40	positive*	115.10	1:5
38	August	Czech Republic	Kralovice	240	negative	-	positive*	107.70	1:5
39	August	Czech Republic	Kralovice	240	positive	34.40	positive*	89.80	1:5
40	August	Czech Republic	Kralovice	240	positive	33.70	positive*	109.90	1:5
41	August	Czech Republic	Svetla nad zazavou	240	negative	-	positive*	208.50	1:5
42	August	Czech Republic	Svetla nad zazavou	240	positive	37.10	positive*	63.50	1:5
43	March	Czech Republic	Český Krumlov	240	negative	-	positive	260.17	cc
44	July	Czech Republic	Recice	250	negative	-	positive	472.10	cc
45	August	Czech Republic	Mirkov	250	negative	-	positive	181.10	cc
46	March	Czech Republic	Volenice	260	positive	35.20	positive	121.01	cc
47	May	Czech Republic	Driten	260	positive	34.40	positive	468.20	cc
48	May	Czech Republic	Chotebor	260	positive	33.30	positive	434.40	cc
49	July	Czech Republic	Netonice	260	positive	32.60	positive	762.90	cc
50	March	Czech Republic	Dacice	280	negative	-	positive	216.69	cc
51	March	Czech Republic	Katovice	300	negative	-	positive	262.00	cc
52	March	Czech Republic	Podebrady	300	positive	33.40	positive	254.59	cc
53	March	Czech Republic	Naceradec	300	negative	-	positive	222.54	cc
54	March	Czech Republic	Horni Lhota	300	negative	-	positive	61.07	cc
55	April	Czech Republic	Lhota	300	negative	-	positive	239.20	cc
56	April	Czech Republic	Pacov	300	positive	33.10	positive	254.13	cc
57	May	Czech Republic	Jihlava	300	positive	34.40	positive	468.20	cc
58	July	Czech Republic	Becvary	300	positive	39.30	positive	777.40	cc
59	August	Czech Republic	horsovsky Tyn	300	positive	32.90	positive	284.70	cc
60	March	Czech Republic	dacice	320	positive	37.50	positive	198.53	cc
61	June	Czech Republic	Oslov	340	negative	-	positive	180.20	cc
62	March	Czech Republic	Malec	350	positive	36.20	positive	199.64	cc
63	March	Czech Republic	novosedl	350	positive	36.20	positive	253.91	cc
64	April	Czech Republic	Domamysl	350	negative	-	positive	254.58	cc
65	May	Czech Republic	Vod hvozdany	350	positive	35.60	positive	429.50	cc
66	June	Czech Republic	Potehy	350	negative	-	positive	212.10	cc
67	July	Czech Republic	Všestary	350	negative	-	positive	785.90	cc
68	July	Czech Republic	Rancirov	350	negative	-	positive	598.50	cc
69	October	Czech Republic	Lanškroun	350	negative	-	positive*	70.10	1:5
70	October	Czech Republic	Lanškroun	350	negative	-	positive*	96.60	1:5
71	November	Czech Republic	Hranice n.Mor.	350	negative	-	positive*	50.50	1:5
72	November	Czech Republic	Hranice n.Mor.	350	negative	-	positive*	53.70	1:5
73	April	Czech Republic	Kluky	360	negative	-	positive	254.58	cc
74	April	Czech Republic	Bohunice	360	negative	-	positive	254.58	cc
75	March	Czech Republic	Vrazdovi Lhotice	400	positive	37.50	positive	224.32	cc
76	March	Czech Republic	dacice	400	positive	29.50	positive	215.08	cc
77	July	Czech Republic	Veselská Lhota	400	positive	34.40	positive	437.20	cc
78	September	Czech Republic	Olomouc	400	negative	-	positive*	104.10	1:5
79	September	Czech Republic	Olomouc	400	negative	-	positive*	104.50	1:5
80	September	Czech Republic	Ústí n.Orlicí	400	negative	-	positive*	78.60	1:5

81	October	Czech Republic	Bystřice n.Perns.	400	negative	-	positive*	190.30	1:5
82	October	Czech Republic	Bystřice n.Perns.	400	negative	-	positive*	176.90	1:5
83	October	Czech Republic	Žďár.n.Sáz.	400	negative	-	positive*	196.50	1:5
84	October	Czech Republic	Žďár.n.Sáz.	400	negative	-	positive*	124.30	1:5
85	May	Czech Republic	Horice	410	positive	39.30	positive	361.30	cc
86	June	Czech Republic	Senagro	410	negative	-	positive	233.50	cc
87	June	Czech Republic	Kosetice	420	negative	-	positive	313.40	cc
88	June	Czech Republic	Horice kobevovice	430	negative	-	positive	269.10	cc
89	March	Czech Republic	Kosova Hora	450	negative	-	positive	197.86	cc
90	July	Czech Republic	Horepnik	450	positive	34.30	positive	220.10	cc
91	August	Czech Republic	Jihlava	450	positive	36.00	positive	246.60	cc
92	August	Czech Republic	Jihlava	450	positive	34.40	positive	213.90	cc
93	August	Czech Republic	Jihlava	450	positive	34.40	positive	213.90	cc
94	August	Czech Republic	Jihlava	450	positive	35.60	positive	458.70	cc
95	August	Czech Republic	Jihlava	450	positive	39.30	positive*	94.50	1:5
96	August	Czech Republic	Jihlava	450	positive	32.10	positive*	69.10	1:5
97	August	Czech Republic	Jihlava	450	positive	32.60	positive*	58.40	1:5
98	September	Czech Republic	Uh.Brod	450	negative	-	positive*	93.40	1:5
99	September	Czech Republic	Olomouc	450	negative	-	positive*	87.80	1:5
100	September	Czech Republic	Olomouc	450	negative	-	positive*	104.10	1:5
101	September	Czech Republic	Šumperk	450	negative	-	positive*	221.70	1:5
102	September	Czech Republic	Svitavy	450	negative	-	positive*	45.20	1:5
103	September	Czech Republic	Ústí n.Orlicí	450	negative	-	positive*	120.90	1:5
104	September	Czech Republic	Ústí n.Orlicí	450	negative	-	positive*	90.90	1:5
105	September	Czech Republic	Uh.Brod	450	negative	-	positive*	49.80	1:5
106	September	Czech Republic	Uh.Brod	450	negative	-	positive*	133.20	1:5
107	September	Czech Republic	Přerov	480	negative	-	positive*	82.20	1:5
108	September	Czech Republic	Olomouc	480	negative	-	positive*	110.10	1:5
109	September	Czech Republic	Šumperk	470	negative	-	positive*	128.40	1:5
110	September	Czech Republic	Šumperk	420	negative	-	positive*	95.10	1:5
111	September	Czech Republic	Ústí n.Orlicí	450	negative	-	positive*	78.60	1:5
112	September	Czech Republic	Ústí n.Orlicí	450	negative	-	positive*	79.10	1:5
113	October	Czech Republic	Žamberk	480	negative	-	positive*	74.80	1:5
114	March	Czech Republic	Vacek	510	positive	33.80	positive	240.45	cc
115	April	Czech Republic	Osek	510	negative	-	positive	78.32	cc
116	May	Czech Republic	Prikosice	510	positive	36.00	positive	422.80	cc
117	May	Czech Republic	Rakova	510	positive	34.90	positive	398.70	cc
118	May	Czech Republic	Dublovice	510	positive	34.40	positive	429.10	cc
119	June	Czech Republic	Zakava	510	negative	-	positive	204.40	cc
120	May	Czech Republic	Kosetice	540	positive	33.70	positive	368.10	cc
121	March	Czech Republic	petrovice	550	positive	31.60	positive	254.04	cc
122	April	Czech Republic	Pisecne	550	positive	36.90	positive	254.58	cc
123	June	Czech Republic	Cicov	550	negative	-	positive	445.20	cc
124	May	Czech Republic	Priseha	600	negative	-	positive	369.70	cc
125	May	Czech Republic	Jihlava	600	positive	36.40	positive	437.40	cc

126	July	Czech Republic	Jirice	600	positive	32.10	positive	676.10	cc
127	August	Czech Republic	Jihlava	600	positive	32.90	positive*	50.50	1:5
128	August	Czech Republic	Jihlava	600	positive	34.90	positive*	53.70	1:5
129	August	Czech Republic	Jihlava	600	positive	33.30	positive*	102.60	1:5
130	September	Czech Republic	Kroměříž	600	negative	-	positive*	119.60	1:5
131	September	Czech Republic	Kroměříž	600	negative	-	positive*	92.10	1:5
132	November	Czech Republic	Ostrava	600	negative	-	positive*	47.10	1:5
133	November	Czech Republic	Ostrava	600	negative	-	positive*	58.40	1:5
134	October	Czech Republic	Lanškroun	650	negative	-	positive*	78.10	1:5
135	October	Czech Republic	Lanškroun	650	negative	-	positive*	101.90	1:5
136	October	Czech Republic	Žďár n.Sáz.	650	negative	-	positive*	119.10	1:5
137	October	Czech Republic	Žďár n.Sáz.	650	negative	-	positive*	180.20	1:5
138	October	Czech Republic	Žďár n.Sáz.	650	negative	-	positive*	175.90	1:5
139	October	Czech Republic	Žďár n.Sáz.	650	negative	-	positive*	75.20	1:5
140	March	Czech Republic	Zhorec	700	positive	34.70	positive	210.33	cc
141	August	Czech Republic	Srby	700	positive	34.30	positive	284.70	cc
142	October	Czech Republic	Vamberk	700	negative	-	positive*	79.40	1:5
143	October	Czech Republic	N.Jičín	700	negative	-	positive*	190.20	1:5
144	October	Czech Republic	N.Jičín	700	negative	-	positive*	92.90	1:5
145	October	Czech Republic	N.Jičín	750	negative	-	positive*	50.10	1:5
146	September	Czech Republic	Vyškov	800	negative	-	positive*	85.90	1:5
147	April	Czech Republic	Podmyce	850	positive	32.50	positive	254.58	cc
148	September	Czech Republic	Chropyně	1000	negative	-	positive*	88.60	1:5
149	October	Czech Republic	N.Jičín	1000	negative	-	positive*	104.50	1:5
150	May	Czech Republic	Uhrinovice	1010	positive	32.60	positive	357.30	cc
151	July	Czech Republic	Holice	1200	positive	36.90	positive	364.90	cc
152	March	Hungary	Jászdózsa	100	negative	-	positive	8,09 / 151,90	1:50 / cc
153	June	Hungary	Zalaszentiván	170	positive	34.58	positive	89.34	1:50
154	March	Hungary	Mórichida	200	negative	-	positive	0,00/ 4,51	1:50 / cc
155	September	Hungary	Nemesgörzsöny	200	negative	-	negative	0,00/7,63	1:50/cc
156	February	Hungary	Dunagyöngye	220	negative	-	negative	0,00/11,90	1:50
157	October	Hungary	Berettyóújfalu	225	positive	36.44	positive	94.44	1:50
158	March	Hungary	Esztár	240	negative	-	negative	0,84/10,97	1:50/cc
159	March	Hungary	Sárvár	260	negative	-	positive	59.49	1:50
160	September	Hungary	Csót	260	positive	36.07	positive	71.43	1:50
161	April	Hungary	Csomád	300	negative		positive	65.90	1:50
162	March	Hungary	Csorvás	320	positive	37.29	positive	85.74	1:50
163	June	Hungary	Bélmegyer	320	negative	-	positive	78.31	1:50
164	March	Hungary	Szarvas	323	negative	-	positive	36,84 / 266,22	1:50 / cc
165	February	Hungary	Hajdúböszörmény	345	negative	-	positive	78.90	1:50
166	March	Hungary	Kisdombegyház	370	negative	-	positive	97.87	1:50
167	March	Hungary	Celldömölk	380	negative	-	positive	35.00	1:50
168	March	Hungary	Kiskunfélegyháza	380	negative	-	positive	29,12 / 272,23	1:50 / cc
169	May	Hungary	Emőd	380	negative	-	positive	53.25	1:50
170	February	Hungary	Hajdúböszörmény	390	positive	36.49	positive	67.10	1:50

171	March	Hungary	Orosháza	400	negative	-	positive	81.99	1:50
172	May	Hungary	Hajdúdorog	400	positive	33.48	positive	73.77	1:50
173	July	Hungary	Cibakháza	400	positive	35.32	positive	88.47	1:50
174	March	Hungary	Paks	410	positive	34.54	positive	52.21	1:50
175	April	Hungary	Geresdlak	410	negative	-	positive	40,10 / 284,46	1:50 / cc
176	May	Hungary	Kocs	410	positive	30.97	positive	98.34	1:50
177	March	Hungary	Tiszavasvári	411	negative	-	positive	64.41	1:50
178	March	Hungary	Borjád	415	negative	-	positive	72.35	1:50
179	October	Hungary	Berettyóújfalu	434	positive	30.64	positive	168.92	1:50
180	February	Hungary	Jászberény	440	positive	34.65	positive	64.90	1:50
181	April	Hungary	Somberek	440	negative	-	positive	72.47	1:50
182	March	Hungary	Tarhos	450	negative	-	positive	56.69	1:50
183	May	Hungary	Tiszakeszi	450	negative	-	positive	77.97	1:50
184	June	Hungary	Tiszanána	450	negative	-	positive	76.38	1:50
185	February	Hungary	Jászkísér	460	negative	-	positive	102.91	1:50
186	October	Hungary	Hajdúszboszló	462	positive	34.78	positive	22.99	1:50
187	February	Hungary	Csanádpalota	470	negative	-	positive	43,50 / 473,03	1:50 / cc
188	March	Hungary	Kétsoprony	480	negative	-	positive	48.38	1:50
189	March	Hungary	Berkesd	490	negative	-	positive	77.70	1:50
190	September	Hungary	Devecser	490	positive	31.06	positive	73.41	1:50
191	March	Hungary	Nagykőrös	510	negative	-	positive	17,20 / 332,13	1:50 / cc
192	March	Hungary	Nyírtelek	520	negative	-	positive	31,18 / 285,92	1:50 / cc
193	February	Hungary	Dombrád	530	negative	-	positive	135.50	1:50
194	February	Hungary	Mosdós	550	positive	35.12	positive	87.20	1:50
195	February	Hungary	Alattyán	550	negative	-	positive	50.90	1:50
196	March	Hungary	Dömsöd	550	negative	-	positive	109.26	1:50
197	March	Hungary	Bödönhát	550	positive	31.28	positive	94.56	1:50
198	May	Hungary	Szarvas	550	positive	34.20	positive	85.90	1:50
199	July	Hungary	Orosháza	550	positive	29.46	positive	71.52	1:50
200	September	Hungary	Veszprémvarsány	557	positive	35.61	positive	71.03	1:50
201	March	Hungary	Gecse	560	negative	-	positive	61.18	1:50
202	February	Hungary	Debrecen	570	negative	-	positive	47.80	1:50
203	February	Hungary	Kenézlő	580	negative	-	positive	219.80	1:50
204	March	Hungary	Földes	590	negative	-	positive	78.46	1:50
205	February	Hungary	Hódmezővásárhely	600	negative	-	positive	48.40	1:50
206	March	Hungary	Rábapordány	600	positive	32.06	positive	78.31	1:50
207	March	Hungary	Füzesgyarmat	610	negative	-	positive	57.72	1:50
208	March	Hungary	Túrkeve	610	positive	38.31	positive	86.25	1:50
209	February	Hungary	Hódmezővásárhely	630	negative	-	positive	27,50 / 430,45	1:50 / cc
210	February	Hungary	Nagyhegyes	650	negative	-	positive	124.50	1:50
211	February	Hungary	Debrecen	650	negative	-	positive	68.60	1:50
212	February	Hungary	Tisztaberek	650	negative	-	positive	63.20	1:50
213	March	Hungary	Nagymágocs	650	positive	36.28	positive	68.53	1:50
214	June	Hungary	Sarud	650	negative	-	positive	55.37	1:50
215	October	Hungary	Harsány	659	positive	33.45	positive	40.57	1:50

216	March	Hungary	Derecske	670	negative	-	positive	45.22	1:50
217	March	Hungary	Szekszárd	680	negative	-	positive	71.54	1:50
218	March	Hungary	Telekgerendás	690	negative	-	positive	91.47	1:50
219	March	Hungary	Szentes	691	negative	-	positive	44.41	1:50
220	June	Hungary	Kisbajcs	700	negative	-	positive	48.72	1:50
221	February	Hungary	Tura	700	negative	-	positive	122.81	1:50
222	October	Hungary	Berettyóújfalu	706	negative	-	positive	18,65/422,88	1:50/cc
223	February	Hungary	Mohács	710	positive	36.24	positive	87.50	1:50
224	March	Hungary	Nyírvár	720	negative	-	positive	44.78	1:50
225	February	Hungary	Mocsa	750	negative	-	positive	125.70	1:50
226	February	Hungary	Komárom	750	negative	-	positive	136.90	1:50
227	March	Hungary	Deszk	750	negative	-	positive	35,44 / 278,48	1:50 / cc
228	April	Hungary	Hegykő	750	positive	35.95	positive	77.72	1:50
229	May	Hungary	Balogszeg	750	negative	-	positive	45.80	1:50
230	July	Hungary	Zsadány	750	positive	34.82	positive	23.42	1:50
231	February	Hungary	Tedej	750	negative	-	positive	108.00	1:50
232	May	Hungary	Perkáta	760	positive	31.27	positive	89.30	1:50
233	September	Hungary	Malomsok	780	positive	33.14	positive	94.31	1:50
234	February	Hungary	Károlyháza	800	positive	34.71	positive	77.50	1:50
235	February	Hungary	Bicsérd	800	negative	-	positive	44,90 / 505,55	1:50 / cc
236	February	Hungary	Kondoros	800	negative	-	positive	39,20 / 469,16	1:50 / cc
237	February	Hungary	Gyula	800	negative	-	positive	79.60	1:50
238	March	Hungary	Marcalgergelyi	800	negative	-	positive	42.21	1:50
239	March	Hungary	Biharnagybajom	800	negative	-	positive	48.46	1:50
240	May	Hungary	Bonyhád	800	positive	36.86	positive	43.11	1:50
241	May	Hungary	Miklós major	800	positive	32.79	positive	71.32	1:50
242	February	Hungary	Besenyeszög	810	positive	33.05	positive	128.14	1:50
243	February	Hungary	Dunaszentgyörgy	830	positive	33.68	positive	49.10	1:50
244	March	Hungary	Fábiánsebestyén	830	negative	-	positive	46.84	1:50
245	February	Hungary	Örménykút	845	negative	-	positive	59.70	1:50
246	March	Hungary	Tass	850	negative	-	positive	76.54	1:50
247	October	Hungary	Hatvan-Nagygombos	850	positive	34.84	positive	47.35	1:50
248	April	Hungary	Mosonszolnok	870	positive	35.06	positive	28,08 / 266,86	1:50 / cc
249	October	Hungary	Balmazújváros	875	positive	37.95	positive	84.66	1:50
250	February	Hungary	Dávod	890	negative	-	positive	90.30	1:50
251	February	Hungary	Bugyi	900	positive	34.66	positive	65.40	1:50
252	May	Hungary	Hódmezővásárhely	900	positive	33.82	positive	78.92	1:50
253	February	Hungary	Nagyecsed	930	positive	36.55	positive	33,80 / 459,61	1:50 / cc
254	February	Hungary	Törtel	960	negative	-	positive	109.80	1:50
255	February	Hungary	Mezőhegyes	960	positive	35.08	positive	76.60	1:50
256	March	Hungary	Hottó	970	negative	-	positive	47.43	1:50
257	March	Hungary	Beled	1050	negative	-	positive	73.75	1:50
258	February	Hungary	Jászladány	1060	negative	-	positive	40,80 / 474,71	1:50 / cc
259	February	Hungary	Sárospatak	1070	negative	-	positive	69.40	1:50

260	February	Hungary	Nyírbátor	1070	positive	35.97	positive	28,50 / 452,65	1:50 / cc
261	March	Hungary	Ikrény	1080	negative	-	positive	23,90 / 246,52	1:50 / cc
262	May	Hungary	Nagyszentjános	1125	negative	-	positive	45.09	1:50
263	February	Hungary	Békés	1150	negative	-	positive	55.30	1:50
264	May	Hungary	Seregélyes	1150	positive	35.21	positive	80.98	1:50
265	September	Hungary	Magyarország	1156	positive	35.91	positive	129.23	1:50
266	March	Hungary	Jászapáti	1200	negative	-	positive	45.74	1:50
267	May	Hungary	Ráckeresztúr	1200	negative	-	positive	9,59/215,10	1:50/cc
268	March	Hungary	Szil	1250	positive	30.96	positive	89.49	1:50
269	March	Hungary	Városföld	1265	negative	-	positive	96.62	1:50
270	March	Hungary	Nemesszalók	1300	negative	-	positive	45.74	1:50
271	March	Hungary	Komárom	1400	positive	30.14	positive	81.32	1:50
272	February	Hungary	Csaholc	1450	negative	-	positive	218.60	1:50
273	February	Hungary	Hódmezővásárhely	1500	negative	-	positive	51.70	1:50
274	February	Hungary	Kazsok	1900	positive	33.47	positive	65.50	1:50
275	February	Hungary	Hajdúböszörmény	2000	negative	-	positive	75.20	1:50
276	February	Hungary	Hajdúnánás	2150	positive	36.50	positive	110.40	1:50
277	February	Hungary	Csipőtelek	2700	positive	30.59	positive	109.80	1:50
278	October	Serbia	Zenta	50	negative	-	negative	0,00/6,75	1:50/cc
279	October	Serbia	Zenta	50	negative	-	negative	0,00/15,63	1:50/cc
280	October	Serbia	Törökkanizsa	50	negative	-	negative	0,00/6,25	1:50/cc
281	October	Serbia	Csantavér	50	negative	-	negative	0,00/12,75	1:50/cc
282	October	Serbia	Csantavér	50	negative	-	negative	0,00/11,00	1:50/cc
283	October	Serbia	Orom	52	negative	-	negative	0,00/11,00	1:50/cc
284	October	Serbia	Ada	52	positive	37.74	positive	3,97/95,25	1:50/cc
285	October	Serbia	Magyarkanizsa	55	positive	38.07	positive	37.96	1:50
286	October	Serbia	Csóka	55	negative	-	positive	30.82	1:50
287	October	Serbia	Gunaras	58	positive	38.77	positive	29.23	1:50
288	October	Serbia	Temerin	100	negative	-	positive	-0,40/594,33	1:50/cc
289	October	Serbia	Mol	100	negative	-	positive	157.16	1:50
290	October	Serbia	Becej	150	positive	35.22	positive	149.25	1:50
291	October	Serbia	Dimitrovgrad	150	negative	-	negative	5,61/7,87	1:50/cc
292	October	Serbia	Malo Crinice	150	negative	-	positive	106.11	1:50
293	October	Serbia	Zagubica	150	negative	-	positive	0,60/64,09	1:50/cc
294	October	Serbia	Cantavir	250	negative	-	positive	36.54	1:50
295	October	Serbia	Backo Gradiste	250	negative	-	positive	61.66	1:50
296	October	Serbia	Lukicevo	270	negative	-	positive	-0,20/665,98	1:50/cc
297	October	Serbia	Dimitrovgrad	300	positive	34.31	positive	-0,40/297,48	1:50/cc
298	October	Serbia	Knic	450	positive	37.80	positive	43.64	1:50
299	October	Serbia	Vrbas	1000	positive	34.79	positive	118.22	1:50
300	October	Serbia	Becej	1600	positive	33.70	positive	62,86	1:50
301	October	Serbia	Padinska Skela	2600	negative	-	positive	16,62/643,46	1:50/cc
302	September	Slovakia	Poltár	100	negative	-	negative	0,00/3,50	1:50/cc
303	September	Slovakia	Banska Bystrica	100	negative	-	negative	0,00/16,50	1:50/cc
304	September	Slovakia	Muráň	100	negative	-	negative	0,00/4,88	1:50/cc

305	September	Slovakia	Levoča	105	negative	-	negative	0,00/9,50	1:50/cc
306	September	Slovakia	Prešov	106	positive	35.06	positive	160.45	1:50
307	September	Slovakia	Žilina	130	negative	-	positive	83.60	1:50
308	September	Slovakia	Bánovce nad Bebravou	130	positive	31.73	positive	189.15	1:50
309	September	Slovakia	Fiľakovo	140	negative	-	positive	2,12/91,25	1:50/cc
310	September	Slovakia	Lučenec	160	positive	33.46	positive	139.29	1:50
311	September	Slovakia	Štúrovo	180	negative	-	negative	0,00/10,38	1:50/cc
312	September	Slovakia	Vráble	200	positive	34.48	positive	358.73	1:50
313	September	Slovakia	Brezno	200	negative	-	positive	56.61	1:50
314	September	Slovakia	Sabinov	200	positive	33.35	positive	100.26	1:50
315	September	Slovakia	Krompachy	200	positive	36.88	positive	17,33/428,88	1:50/cc
316	October	Slovakia	Námestovo	200	positive	31.14	positive	32.76	1:50
317	October	Slovakia	Námestovo	200	positive	30.96	positive	152.13	1:50
318	September	Slovakia	Trnava	205	positive	32.14	positive	87.70	1:50
319	September	Slovakia	Sliač	210	negative	-	positive	76.98	1:50
320	September	Slovakia	Žiar	225	positive	32.96	positive	411.77	1:50
321	February	Slovakia	Moldava nad Bodvou	240	negative	-	positive	12,80 / 356,26	1:50 / cc
322	September	Slovakia	Trebišov	250	positive	31.34	positive	209.26	1:50
323	September	Slovakia	Spišská Nová Ves	250	positive	28.97	positive	236.90	1:50
324	September	Slovakia	Želiezovce	300	positive	33.01	positive	207.94	1:50
325	September	Slovakia	Turňa nad Bodvou	300	positive	31.63	positive	280.95	1:50
326	September	Slovakia	Prešov	300	positive	28.25	positive	93.92	1:50
327	October	Slovakia	Tvrdošín	300	positive	28.81	positive	253.23	1:50
328	September	Slovakia	Velký Krtíš	315	positive	36.45	positive	55.69	1:50
329	February	Slovakia	Šurany	350	negative	-	positive	23,80 / 435,61	1:50 / cc
330	February	Slovakia	Nové Zámky	350	negative	-	positive	47.20	1:50
331	September	Slovakia	Prešov	350	positive	27.50	positive	337.30	1:50
332	September	Slovakia	Jesenské	370	positive	34.01	positive	75.53	1:50
333	September	Slovakia	Topoľčany	380	positive	28.77	positive	82.41	1:50
334	February	Slovakia	Košice	400	positive	34.36	positive	133.60	1:50
335	September	Slovakia	Oponice	420	positive	30.19	positive	141.40	1:50
336	September	Slovakia	Nitra	500	positive	36.18	positive	4,50/155,25	1:50/cc
337	September	Slovakia	Partizánske	500	positive	32.10	positive	119.18	1:50
338	September	Slovakia	Komárno	500	positive	36.41	positive	59.92	1:50
339	September	Slovakia	Hlohovec	505	positive	34.76	positive	108.99	1:50
340	September	Slovakia	Trenčín	520	positive	32.43	positive	119.71	1:50
341	February	Slovakia	Dunajská Streda	550	negative	-	positive	66.70	1:50
342	July	Slovakia	Hlohovec	550	positive	33.62	negative	28.41	1:50
343	February	Slovakia	Lučenec	600	negative	-	positive	42,60 / 420,90	1:50 / cc
344	February	Slovakia	Trnava	600	negative	-	positive	82.00	1:50
345	February	Slovakia	Dubník	600	negative	-	positive	129.00	1:50
346	September	Slovakia	Turčianske Teplice	630	positive	36.69	positive	171.56	1:50
347	February	Slovakia	Rožňava	700	positive	34.61	positive	103.20	1:50
348	July	Slovakia	Piešťany	700	positive	34.14	negative	-0.43	1:50

349	September	Slovakia	Krupina	700	positive	33.36	positive	39.17	1:50
350	February	Slovakia	Nové Zámky	900	negative	-	positive	171.40	1:50
351	February	Slovakia	Gabčíkovo	1100	positive	35.29	positive	61.30	1:50
352	February	Slovakia	Pribeta	1300	negative	-	positive	133.00	1:50
353	February	Slovakia	Trnava	2500	negative	-	positive	23,20 / 395,23	1:50 / cc
354	February	Slovakia	Bratislava	2700	negative	-	positive	32,70 / 502,71	1:50 / cc
355	October	Slovenia	Murska Sobota Obmocje	50	negative	-	negative	0,48/24,5	1:50/cc
356	October	Slovenia	Murska Sobota Obmocje	55	negative	-	negative	1,25/10,08	1:50/cc
357	October	Slovenia	Murska Sobota Obmocje	55	negative	-	negative	-0,77/10,38	1:50/cc
358	October	Slovenia	Murska Sobota Obmocje	50	negative	-	negative	-0,30/3,93	1:50/cc
359	October	Slovenia	Vodice	50	negative	-	positive	64.83	1:50
360	October	Slovenia	Vodice	50	negative	-	positive	16,42/300,6	1:50/cc
361	October	Slovenia	Murska Sobota Obmocje	55	negative	-	negative	-0,67/4,13	1:50/cc
362	October	Slovenia	Murska Sobota Obmocje	80	negative	-	negative	-0,67/15,73	1:50/cc
363	October	Slovenia	Vodice	80	positive	34.04	positive	157.10	1:50
364	October	Slovenia	Kranj/Farma Hrastje	150	positive	31.47	positive	26.66	1:50
365	October	Slovenia	Kranj/Farma Cerklje	160	positive	35.15	positive	14,39/304,93	1:50/cc
366	October	Slovenia	Kranj/Farma Zabnica	160	positive	32.84	positive	82.12	1:50
367	May	Slovenia	Kocevje	250	positive	28.80	positive	92.16	1:50
368	May	Slovenia	Kocevje	250	positive	32.70	positive	57.77	1:50
369	October	Slovenia	PoljCe/Bled	360	positive	31.74	positive	98.55	1:50
370	May	Slovenia	Stara Cerkev	400	positive	36.89	positive	79.79	1:50

aID Screen® Q Fever Indirect Multi-species kit (IDVet Inc., Grabels, France) was used to test the majority of the samples; cut-off value for diluted samples (1:50 dilution) was S/P % > 20, cut-off value for non-diluted milk samples was S/P % > 40

*Tested with IDEXX Q Fever Ab Test kit (IDEXX Europe B.V., Hoofddorp, the Netherlands); cut-off value for diluted samples (1:5 dilution) was S/P % > 30

cc: non-diluted milk sample was used for the ELISA

Table S2. Backround information of Elisa and CFT Phase I and Phase II results of pregnant and cows with pregnancy loss checked by Biopryn test and transrectal palpation on day 60-70 after AI.

Sample ID	NO of AI	Biopryn test results	Transrectal palpation days 60-70 after AI	ELISA results	ELISA S/P %	CFT Phase I results		CFT phase II results	
1	1	pregnant	pregnant	positive	279.58		negative	1:10 ++	positive
2	1	pregnant	pregnant	negative	25.49				
3	1	pregnant	pregnant	positive	362.68	1:20 ++	positive	1:40 ++	positive
4	4	pregnant	pregnant	negative	18.13				
5	2	pregnant	pregnant	negative	0.00				
6	1	pregnant	pregnant	positive	302.68		negative	-	negative
7	2	pregnant	pregnant	negative					
8	3	pregnant	pregnant	negative	14.19				
9	1	open		positive	203.94		negative	1:20 +	positive
10	1	pregnant	pregnant	positive	141.27	-	negative	-	negative
11	1	pregnant	open	positive	512.82	1:20 ++	positive	1:20 ++	positive
12	1	open		positive	510.00	1:40 +++	positive	1:160 ++	positive
13	4	pregnant	pregnant	negative	16.83				
14	1	pregnant	pregnant	negative	0.00				
15	2	pregnant	pregnant	negative	14.79				
16	1	pregnant	pregnant	positive	288.45	1:20 ++	positive		negative
17	3	pregnant	open	positive	44.93	-	negative	-	negative
18	1	pregnant	open	negative	0.99				
19	1	pregnant	pregnant	negative	0.00				
20	1	pregnant	pregnant	negative	0.00				
21	2	pregnant	open	positive	328.17	1:20 ++	positive	1:20 +	positive
22	2	open		positive	382.11	1:80 +	positive	1:160 +	positive
23	2	pregnant	pregnant	negative	0.00				
24	2	open		negative	0.00				
25	1	pregnant	pregnant	negative	8.73				
26	7	pregnant	pregnant	positive	54.23	-	negative	-	negative
27	5	pregnant	pregnant	positive	351.69	1:10 +++	positive	1:20 +	positive
28	1	pregnant	pregnant	negative	0.00				
29	5	pregnant	pregnant	positive	298.59	1:40 +	positive	1:80 ++	positive
30	1	open		positive	348.87	1:20 ++	positive	1:80 +++	positive
31	2	pregnant	pregnant	negative	17.58				
32	1	pregnant	pregnant	positive	210.70	-	negative	1:20 +	positive
33	2	open		positive	112.20				
34	1	pregnant	pregnant	negative	24.37				
35	1	pregnant	pregnant	positive	228.72	-	negative	-	negative
36	1	pregnant	pregnant	positive	53.36	-	negative	1:20 +	positive
37	1	pregnant	pregnant	negative	25.21				

38	1	pregnant	pregnant	positive	230.81	-	negative	1:20 +	positive
39	1	pregnant	open	positive	339.91	1:20 ++	positive	1:160 ++	positive
40	2	pregnant	pregnant	negative	6.45				
41	1	pregnant	open	positive	350.05	1:80 +	positive	1:160 +	positive
42	3	open		negative	23.43				
43	1	open		positive	321.23	1:10 ++	positive	1:40 +	positive
44	4	pregnant	pregnant	positive	386.45	1:320 +	positive	1:160 +	positive
45	2	pregnant	open	positive	183.70	-	negative		negative
46	1	pregnant	pregnant	negative	4.83				
47	2	open		negative	20.12				
48	2	pregnant	open	positive	359.53	1:20 ++	positive	1:40 ++	positive
49	4	pregnant	open	negative	29.29				
50	1	pregnant	pregnant	negative	23.51				
51	2	pregnant	open	positive	243.60	-	negative	-	negative
52	2	pregnant	pregnant	negative	29.23				
53	3	open		negative	7.25				
54	4	pregnant	pregnant	negative	18.12				
55	1	pregnant	open	positive	317.44	1:10 +++	positive	1:10 +++	positive
56	2	open		positive	381.71	1:40 +++	positive	1:80 +	positive
57	1	open		positive	226.92	-	negative	-	negative
58	1	open		negative	17.44				
59	3	pregnant	pregnant	negative	16.14				
60	4	open		negative	10.32				
61	1	pregnant	open	positive	266.45	1:20 +	positive	1:20 +	positive
62	1	pregnant	pregnant	positive	331.66	1:20 ++	positive	1:80 +	positive
63	2	pregnant	pregnant	positive	324.27	1:20 ++	positive	1:40 ++	positive
64	2	pregnant	pregnant	positive	259.05	1:20 +	positive	1:40 +	positive
65	1	pregnant	open	positive	106.54	-	negative		negative
66	5	open		positive	229.19	-	negative	1:20 +	positive
67	2	open		negative	11.37				
68	4	open		negative	6.83				
69	2	pregnant	pregnant	negative	18.88				
70	3	pregnant	open	positive	233.27	1:10 +	positive	1:20 +++	positive
71	1	open		negative	12.32				
72	2	pregnant	pregnant	negative	5.21				
73	4	pregnant	pregnant	positive	127.58	-	negative	1:20 +	positive
74	2	pregnant	pregnant	positive	92.04	-	negative	1:20 +	positive
75	2	pregnant	pregnant	positive	265.59	-	negative	1:20 +	positive
76	1	pregnant	open	positive	251.28	1:10 ++++	positive	1:20 ++	positive
77	1	pregnant	pregnant	positive	170.81		negative	1:20 ++	positive
78	2	open		negative	18.39				
79	1	pregnant	open	positive	154.88	1:10 +	positive	1:160 +	positive
80	2	open		negative	32.42				
81	3	open		negative	6.82				
82	1	pregnant	pregnant	positive	209.19	1:10 ++	positive	1:20 +	positive

83	1	pregnant	open	positive	203.89	1:10 ++	positive	1:80 ++	positive
84	1	open		negative	49.00				
85	2	pregnant	pregnant	positive	291.94	1:20 ++	positive	1:160 +	positive
86	2	pregnant	pregnant	negative	18.12				
87	1	pregnant	open	positive	87.76	-	negative	-	negative
88	1	open		negative	24.94				
89	1	pregnant	pregnant	negative	18.35				
90	1	pregnant	pregnant	negative	8.00				
91	1	pregnant	pregnant	negative	5.41				
92	1	pregnant	pregnant	negative	2.59				
93	1	pregnant	pregnant	positive	451.76	1:10 +++	positive	-	negative
94	2	pregnant	pregnant	positive	209.65	-	negative	1:40 +	positive
95	4	pregnant	pregnant	positive	386.59		negative	1:20 ++	positive
96	2	open		positive	556.24	1:20 ++	positive	1:40 +	positive
97	1	open		negative	15.29				
98	1	pregnant	pregnant	negative	5.88				
99	2	pregnant	open	negative	2.82				
100	1	pregnant	pregnant	negative	1.65				
101	1	pregnant	pregnant	negative	8.94				
102	3	open		negative	30.20				
103	2	pregnant	pregnant	negative	10.24				
104	4	pregnant	pregnant	negative	25.81				
105	3	open		negative	40.20				
106	3	pregnant	open	positive	230.59	-	negative	1:80 +	positive
107	1	pregnant	pregnant	negative	32.47				
108	3	pregnant	pregnant	negative	14.82				
109	2	open		negative	33.41				
110	3	open		negative	21.41				
111	1	pregnant	pregnant	positive	322.12		negative	1:10 ++	positive
112	1	pregnant	pregnant	positive	379.53		negative	1:80 +	positive
113	3	open		positive	193.80	1:10 +++	positive	1:10 ++	positive
114	1	open		negative	9.30				
115	1	open		positive	73.10	-	negative	-	negative
116	1	pregnant	pregnant	negative	45.63				
117	1	pregnant	pregnant	negative	47.32				
118	1	open		positive	67.75	-	negative	-	negative
119	7	open		positive	489.72	1:160 +	positive	1:320 ++	positive
120	1	open		negative	3.24				
121	2	open		positive	98.40				
122	5	pregnant	pregnant	positive	292.25	1:10 ++	positive	-	negative
123	3	open		negative	25.20				
124	3	pregnant	pregnant	positive	214.65	1:20++	positive	-	negative
125	1	open							
126	3	pregnant	pregnant	positive	400.14	1:40 +++	positive	1:320 ++	positive
127	1	pregnant	pregnant	negative	47.75				

128	1	open		negative	7.35				
129	2	pregnant	pregnant	positive	335.77	1:20 ++	positive	1:40 +	positive
130	4	open		positive	118.20				
131	1	pregnant	open	positive	486.62	1:20 +++	positive	1:20 +	positive
132	2	pregnant	pregnant	positive	106.06	-	negative	1:10 ++	positive
133	1	pregnant	pregnant	negative	7.75				
134	3	open		positive	531.55	1:80 ++	positive	1:80 +	positive
135	3	open		negative	4.93				
136	1	pregnant	pregnant	negative	4.79				
137	3	pregnant	pregnant	positive	204.23	-	negative	1:80 +	positive
138	4	pregnant	pregnant	negative	0.70				
139	2	pregnant	pregnant	negative	1.13				
140	1	pregnant	pregnant	negative	2.68				
141	3	pregnant	pregnant	negative					
142	4	pregnant	pregnant	negative	8.65				
143	1	open		positive	64.74	-	negative	-	negative
144	2	pregnant	pregnant	negative	19.27				
145	1	open		positive	98.30				
146	3	pregnant	open	positive	337.44	1:20 ++++	positive	1:160 +	positive
147	1	open		positive	78.10	-	negative	-	negative
148	1	pregnant	open	positive	288.53	1:20 +	positive	1:20 ++	positive
149	1	open		positive	120.32				
150	1	open		positive	167.18				
151	1	pregnant	pregnant	positive	122.09		negative	1:20 +	positive
152	1	pregnant	pregnant	positive	354.03	1:40 +	positive	1:40 +	positive
153	1	pregnant	pregnant	negative	4.22				
154	2	open		negative	22.12				
155	1	pregnant	pregnant	positive	93.18		negative	1:20 +	positive
156	1	open		positive	142.78				
157	7	pregnant	pregnant	positive	261.14		negative	1:10 +++	positive
158	5	pregnant	pregnant	positive	261.71	1:10 ++	positive	1:20 ++	positive
159	2	open		positive	330.33	1:80 +++	positive	1:160 ++	positive
160	6	open		positive	344.64	1:40 +++	positive		negative
161	4	pregnant	pregnant	negative	9.14				
162	1	open		positive	313.08	1:10 ++	positive	1:40 +	positive
163	2	open		negative	39.32				
164	2	pregnant	pregnant	positive	282.65	-	negative	1:40 +	positive
165	1	pregnant	pregnant	negative	3.22				
166	1	open		positive	221.74				
167	1	pregnant	pregnant	negative	42.37				
168	1	open		positive	140.65				
169	1	open		positive	242.42				
170	7	pregnant	pregnant	positive	332.70	1:20 ++	positive	1:80 +	positive
171	2	open		negative	24.76				
172	1	pregnant	pregnant	positive	341.04	1:80 +	positive	1:80 ++	positive

173	5	pregnant	pregnant	positive	289.76	1:40 ++	positive	1:160 +	positive
174	1	open		positive	65.21	-	negative	-	negative
175	1	pregnant	pregnant	positive	235.36	-	negative	1:80 +	positive
176	3	open		positive	73.55	-	negative	-	negative
177	4	open		negative	1.52				
178	2	pregnant	pregnant	negative	13.74				
179	2	pregnant	pregnant	negative	35.92				
180	1	pregnant	pregnant	positive	114.98	-	negative	-	negative
181	1	pregnant	pregnant	positive	218.82	-	negative	-	negative
182	1	open		positive	102.12		negative		negative
183	1	pregnant	pregnant	positive	261.88		negative	1:20 ++	positive
184	1	pregnant	pregnant	positive	198.59	-	negative		negative
185	2	pregnant	pregnant	positive	378.12	1:10 ++	positive	1:20 ++	positive
186	2	pregnant	pregnant	positive	604.00	1:160 +	positive	1:40 +	positive
187	1	pregnant	pregnant	positive	693.18	1:160 +	positive	1:80 +	positive
188	7	pregnant	pregnant	positive	640.24	1:20 ++	positive	1:160 +	positive
189	3	pregnant	pregnant	negative	20.99				
190	1	open		positive	201.41	-	negative	-	negative
191	4	pregnant	open	positive	274.12	1:10 ++	positive	1:20 +	positive
192	1	pregnant	pregnant	positive	193.88	-	negative	1:80 +	positive
193	1	pregnant	pregnant	positive	87.53	-	negative	-	negative
194	2	open		negative	16.22				
195	2	open		positive	378.35	1:20 +	positive	1:40 +	positive
196	1	pregnant	pregnant	positive	417.65	1:20 +	positive	1:20 +	positive
197	1	pregnant	pregnant	positive	418.59	1:20 ++	positive	1:40 +	positive
198	1	pregnant	pregnant	negative	3.06				
199	3	open		negative	18.57				
200	1	pregnant	pregnant	negative	0.94				
201	2	pregnant	pregnant	positive	419.29	1:80 +	positive	1:80 ++	positive
202	1	open		negative	15.53				
203	4	open		negative	0.56				
204	2	open		positive	95.92	-	negative	-	negative
205	3	pregnant	pregnant	negative	3.66				
206	1	pregnant	pregnant	negative	1.27				
207	3	open		negative	7.83				
208	2	open		negative	8.17				
209	7	pregnant	open	positive	212.03	1:10 +	positive	1:10 +++	positive
210	1	pregnant	open	negative	6.47				
211	1	pregnant	pregnant	negative	0.45				
212	1	open		negative	0.45				
213	3	open		positive	453.80	1:160 +++	positive	1:320 +++	positive
214	4	open		negative	17.93				
215	2	pregnant	pregnant	positive	378.21	1:160 +	positive	1:160 +	positive
216	1	open		negative	2.27				
217	3	open		negative	35.15				

218	2	pregnant	pregnant	positive	94.44	-	negative	1:10 ++	positive
219	2	open		negative	18.75				
220	1	pregnant	pregnant	positive	202.27		negative	1:10 +++	positive
221	1	pregnant	open	positive	394.21	1:160 ++	positive	1:320 +	positive
222	5	open		negative	15.10				
223	4	pregnant	pregnant	positive	358.34	1:80 +	positive	1:320 +	positive
224	4	open		negative	28.38				
225	1	pregnant	pregnant	negative	17.37				
226	2	open		negative	38.22				
227	6	pregnant	pregnant	negative	21.04				
228	2	pregnant	pregnant	positive	196.14	1:20 +	positive	1:40 +	positive
229	4	open		positive	412.71	1:80 +++	positive	1:160 +	positive
230	2	open		negative	16.92				
231	3	open		negative	24.02				
232	4	pregnant	pregnant	positive	124.97	-	negative	1:10 ++	positive
233	3	pregnant	pregnant	positive	87.63	1:10 ++	positive	1:20 +	positive
234	2	pregnant	pregnant	positive	81.04		negative	1:20 +	positive
235	2	open		negative	1.82				
236	2	open		negative	44.95				
237	1	pregnant	pregnant	positive	107.72	-	negative		negative
238	2	pregnant	pregnant	negative	27.24				
239	1	pregnant	pregnant	negative	6.47				
240	3	pregnant	pregnant	positive	233.26	1:20 ++	positive	1:80 ++	positive
241	4	pregnant	open	negative	0.00				
242	2	open		positive	230.08	1:20 ++	positive	1:160 +	positive
243	1	pregnant	pregnant	positive	173.33	1:20 ++	positive	1:160 +++	positive
244	4	pregnant	pregnant	negative	0.68				
245	4	open		positive	84.22	-	negative	-	negative
246	4	pregnant	open	negative	1.59				
247	3	pregnant	open	positive	250.51	1:10 ++	positive	1:320 +	positive
248	4	open		negative	32.56				
249	1	pregnant	pregnant	positive	114.76		negative	1:20 +	positive
250	1	pregnant	pregnant	negative	1.93				
251	1	open		positive	143.36	1:20 ++	positive	1:320 ++	positive
252	1	pregnant	open	positive	159.48	1:10 ++	positive	1:160 +	positive
253	1	open		positive	64.70	-	negative	1:20 +	positive
254	3	open		negative	6.34				
255	1	open		positive	137.80	-	negative	1:20 +	positive
256	1	pregnant	pregnant	positive	139.94	-	negative	-	negative
257	2	pregnant	pregnant	negative	4.51				
258	2	pregnant	open	positive	380.47	1:40 ++	positive	1:40 ++	positive
259	4	open		positive	310.89	1:20 ++	positive	1:20 ++	positive
260	2	pregnant	pregnant	negative	20.22				
261	3	pregnant	pregnant	negative	28.05				
262	3	open		negative	1.28				

263	7	pregnant	pregnant	negative	1.08				
264	2	open		positive	354.17	1:20 ++	positive	1:40 ++	positive
265	2	open		positive	142.89		negative	1:20 +	positive
266	1	pregnant	open	positive	149.95	1:10 +	positive	1:20 ++	positive
267	1	pregnant	pregnant	negative	22.18				
268	1	open		positive	400.59	1:160 +	positive	1:40 ++	positive
269	2	open		negative	2.85				
270	4	pregnant	pregnant	negative	3.43				
271	4	open		positive	220.90		negative	1:20 ++	positive
272	4	open		negative	2.06				
273	1	pregnant	pregnant	positive	323.95	1:20 ++	positive	1:160 ++	positive
274	1	pregnant	pregnant	positive	300.79	1:20 ++	positive	1:80 ++	positive
275	1	pregnant	pregnant	positive	161.24	-	negative	1:20 +	positive
276	2	pregnant	open	negative	2.36				
277	1	pregnant	pregnant	positive	60.75	-	negative	-	negative
278	5	open		positive	448.71	1:10 ++	positive	-	negative
279	1	open		negative	41.88				
280	2	pregnant	pregnant	negative	21.29				
281	5	open		positive	197.41	-	negative	-	negative
282	1	open		negative	12.00				
283	6	pregnant	pregnant	negative	2.59				
284	7	open		negative	0.71				
285	3	pregnant	pregnant	negative	4.71				
286	6	pregnant	pregnant	positive	531.76	1:160 +	positive	1:160 ++	positive
287	4	open		negative	2.35				
288	1	pregnant	pregnant	negative	11.29				
289	1	pregnant	pregnant	positive	165.65	-	negative	-	negative
290	3	open		negative	0.94				
291	1	open		positive	533.65	1:20 ++	positive	1:160 +	positive
292	1	pregnant	pregnant	positive	579.06	1:160 +	positive	1:40 ++	positive
293	2	open		positive	276.71	-	negative	-	negative
294	1	open		positive	805.57	1:20 ++	positive	1:20 ++	positive
295	5	open		negative	-3.28				
296	3	open		positive	303.93	-	negative	1:20 +	positive
297	4	pregnant	pregnant	negative	33.77				
298	1	pregnant	open	positive	883.28	1:160 +	positive	1:320 +	positive
299	1	pregnant	pregnant	positive	537.05		negative	-	negative
300	3	pregnant	pregnant	negative	3.28				
301	2	open		negative	0.66				
302	2	open		negative	1.97				
303	1	open		positive	631.48	1:20 ++	positive	1:40 +	positive
304	6	open		positive	744.92	1:160 ++	positive	1:160 +	positive
305	1	pregnant	pregnant	positive	669.18		negative		negative
306	6	pregnant	pregnant	negative	-3.61				
307	3	pregnant	pregnant	negative	16.07				

308	5	pregnant	pregnant	negative	-2.30				
309	5	pregnant	pregnant	negative	-8.20				
310	5	open		negative	3.93				
311	6	open		negative	-6.56				
312	4	open		negative	-3.93				
313	2	pregnant	pregnant	negative	28.52			-	
314	2	open		positive	301.97				
315	3	pregnant	pregnant	positive	171.48	-	negative	-	negative
316	1	open		positive	268.52	-	negative	1:20 ++	positive
317	2	open		negative	42.95				
318	3	open		positive	58.03	-	negative	-	negative
319	1	open		positive	371.15	-	negative	1:20 ++	positive
320	1	pregnant	open	positive	352.46	-	negative	1:20 ++	positive
321	3	pregnant	pregnant	negative	40.12				

Table S3. Summary of the questionnaire and QF G2 and QF G1 titers among 70 high-risk subjects in Hungary

Occupation	Length of employment	Titer	QF G2	Titer	QF G1	Age	sex
inseminator	2012-	1:128	positive	1:128	positive	48	Male
parlour worker	2019-		negative		negative	54	Male
herd manager	2017-	1:128	positive	1:64	positive	49	Male
animal caretaker	2017-	1:128	positive	1:32	positive	38	Male
inseminator	2019-	1:1024	positive	1:512	positive	28	Male
parlour worker	2007-		negative		negative	55	Male
animal caretaker	2010	1:128	positive	1:64	positive	41	Male
animal caretaker	2000-	1:16	positive	1:32	positive	52	Male
herd manager	2015-	1:16	positive		negative	49	Male
animal caretaker	2017-	1:256	positive	1:256	positive	34	Male
animal caretaker	2010-	1:256	positive	1:256	positive	56	Male
animal caretaker	2017-	1:256	positive	1:128	positive	42	Male
parlour worker	2010-	1:16	positive	1:16	positive	55	Female
veterinarian	2009	1:512	positive	1:1024	positive	58	Male
parlour worker	1989-	1:512	positive	1:1024	positive	62	Male
parlour worker	2010-	1:1024	positive	1:512	positive	47	Male
animal caretaker	2017-	1:16	positive		negative	36	Male
inseminator	2014-	1:256	positive	1:128	positive	48	Male
herd manager	2019-		negative		negative	27	Male
animal caretaker	2019-	1:64	positive	01:32	positive	19	Male
inseminator	2010-	1:256	positive	1:128	positive	47	Male
parlour worker	2010-	1:256	positive	1:128	positive	50	Male
parlour worker	2003-	1:64	positive	01:32	positive	57	Female
animal caretaker	1998-	1:64	positive	01:32	positive	52	Female
veterinarian	2011-	1:128	positive	1:64	positive	42	Male
animal caretaker	1999-	1:128	positive	1:64	positive	51	Male
parlour worker	2016-		negative		negative	50	Female
parlour worker	2018-		negative		negative	35	Female
parlour worker	2004-	1:16	positive	01:16	positive	49	Male
parlour worker	2015-		negative		negative	35	Female
animal caretaker	2017-	1:32	positive	01:16	positive	40	Female
inseminator	2016-	1:16	positive	01:32	positive	64	Male
animal caretaker	2016-	1:16	positive	01:16	positive	48	Male
animal caretaker	2019-	1:16	positive		negative	27	Male
veterinarian	2002	1:128	positive	1:64	positive	48	Male
parlour worker	2020-		negative		negative	56	Female
herd manager	2015-	1:16	positive	01:32	positive	60	Male
parlour worker	2016-		negative		negative	50	Female
inseminator	1979-	1:16	positive	1:16	positive	59	Male
herd manager	1984-		negative		negative	56	Male
animal caretaker	2001-	1:16	positive		negative	60	Female
animal caretaker	2000-	01:32	positive	1:16	positive	48	Female

veterinarian	2011-	1:128	positive	1:64	positive	38	Male
parlour worker	2011-	1:64	positive	01:32	positive	37	Female
inseminator	1989-	1:256	positive	1:128	positive	52	Male
animal caretaker	2001-	1:16	positive		negative	59	Male
veterinarian	2005	1:128	positive	1:64	positive	42	Male
herd manager	2001-	1:16	positive		negative	43	Male
inseminator	1989-	1:64	positive	01:32	positive	52	Male
animal caretaker	1991-	1:128	positive	1:64	positive	49	Male
veterinarian	1999	1:64	positive	1:64	positive	52	Male
animal caretaker	1984	1:128	positive	1:128	positive	64	male
parlour worker	1995	1:128	positive	1:128	positive	55	Female
animal caretaker	2012	1:16	positive	1:16	positive	36	male
animal caretaker	2010	1:512	positive	1:256	positive	40	Male
herd manager	2019	01:32	positive	1:16	positive	21	Male
parlour worker	2019		negative		negative	22	Male
animal caretaker	2006	1:16	positive	1:16	positive	49	male
animal caretaker	2004	1:16	positive	1:16	positive	49	Male
animal caretaker	2015	1:16	positive	1:16	positive	45	Male
inseminator	2015	1:512	positive	1:256	positive	30	Male
parlour worker	2019		negative		negative	30	Male
inseminator	2005	1:64	positive	1:64	positive	38	Male
veterinarian	2003	1:256	positive	1:256	positive	41	Male
animal caretaker	2015	1:16	positive	1:16	positive	30	Male
veterinarian	1994	1:1024	positive	1:512	positive	50	Male
animal caretaker	1990	1:256	positive	1:128	positive	53	Male
animal caretaker	2000	1:128	positive	1:128	positive	52	Male
inseminator	2015	1:256	positive	1:256	positive	30	Male
inseminator	1973	1:64	positive	01:32	positive	66	Male

Table S4. Summary of real-time PCR results of *C. burnetii* in retained and normaly separeted placentas with Ct value and cow parity of studied cows.

Farm	Country	Town	Retained placenta (RP)	Normal (N)	real-time PCR Result	Ct Value	Cow Parity
1	Hungary	Farm1	RP		positive	36.82	3
2	Hungary	Farm1	RP		negative		1
3	Hungary	Farm2		N	positive	36.91	1
4	Hungary	Farm2		N	positive	35.81	1
5	Hungary	Farm2		N	positive	36.54	1
6	Hungary	Farm2	RP		positive	31.25	4
7	Hungary	Farm2	RP		positive	32.21	3
8	Hungary	Farm2		N	positive	36.45	2
9	Hungary	Farm2		N	negative		2
10	Hungary	Farm2	RP		positive	17.56	1
11	Hungary	Farm2	RP		positive	34.28	5
12	Hungary	Farm2		N	negative		1
13	Slovakia	Farm3	RP		positive	36.07	3
14	Slovakia	Farm4	RP		negative		1
15	Slovakia	Farm4	RP		positive	33.86	3
16	Slovakia	Farm4	RP		positive	34.56	5
17	Hungary	Farm5	RP		positive	33.25	3
18	Hungary	Farm5	RP		positive	35.56	1
19	Hungary	Farm5	RP		positive	32.38	4
20	Hungary	Farm5		N	positive	32.29	2
21	Hungary	Farm5		N	positive	36.06	1
22	Hungary	Farm5		N	negative		3
23	Hungary	Farm5		N	positive	36.26	2
24	Hungary	Farm5	RP		positive	34.48	3
25	Hungary	Farm5	RP		positive	18.28	3
26	Hungary	Farm6	RP		negative		2
27	Hungary	Farm6	RP		positive	34.72	4
28	Hungary	Farm6	RP		positive	31.42	6
29	Hungary	Farm7		N	negative		1
30	Hungary	Farm7	RP		positive	34.49	1
31	Hungary	Farm7		N	negative		2
32	Hungary	Farm7	RP		positive	33.26	3
33	Hungary	Farm7	RP		positive	36.24	2
34	Hungary	Farm8	RP		positive	29.67	3
35	Hungary	Farm8	RP		positive	36.82	1
36	Hungary	Farm9		N	negative		1
37	Hungary	Farm9	RP		positive	36.12	4
38	Hungary	Farm9	RP		positive	34.41	3

39	Hungary	Farm9	RP		positive	36.73	1
40	Hungary	Farm9		N	negative		1
41	Hungary	Farm9	RP		positive	32.89	6
42	Hungary	Farm10	RP		positive	36.51	4
43	Hungary	Farm11	RP		positive	35.95	3
44	Hungary	Farm11		N	negative		2
45	Hungary	Farm11	RP		positive	36.47	1
46	Hungary	Farm11		N	negative		1
47	Hungary	Farm11		N	negative		1
48	Hungary	Farm11		N	negative		2
49	Hungary	Farm12		N	negative		1
50	Hungary	Farm12		N	positive	35.08	2
51	Hungary	Farm12		N	negative		2
52	Hungary	Farm12		N	positive	36.32	1
53	Hungary	Farm12		N	negative		3
54	Hungary	Farm12		N	negative		1
55	Hungary	Farm12	RP		positive	33.79	4
56	Hungary	Farm12		N	positive	36.18	2
57	Hungary	Farm12		N	negative		2
58	Hungary	Farm12		N	negative		1
59	Hungary	Farm12		N	negative		2
60	Hungary	Farm12	RP		positive	36.91	3
61	Hungary	Farm13		N	negative		2
62	Hungary	Farm13		N	negative		2
63	Hungary	Farm13		N	negative		1
64	Hungary	Farm13		N	positive	33.11	3
65	Hungary	Farm13	RP		positive	36.52	2
66	Hungary	Farm13		N	negative		2
67	Hungary	Farm13		N	negative		3
68	Hungary	Farm13	RP		positive	36.36	4
69	Hungary	Farm13	RP		positive	36.06	3
70	Hungary	Farm13	RP		negative		2
71	Hungary	Farm13	RP		positive	32.67	1
72	Hungary	Farm14	RP		negative		1
73	Hungary	Farm14	RP		negative		1
74	Hungary	Farm14	RP		positive	19.01	6
75	Hungary	Farm14	RP		positive	18.21	5
76	Hungary	Farm15		N	negative		2
77	Hungary	Farm15		N	negative		2
78	Hungary	Farm15		N	negative		1
79	Hungary	Farm15	RP		positive	35.53	2
80	Hungary	Farm16	RP		positive	20.05	4
81	Hungary	Farm16		N	negative		1

82	Hungary	Farm16		N	negative		1
83	Hungary	Farm16		N	negative		1
84	Hungary	Farm16		N	negative		1
85	Hungary	Farm16	RP		positive	35.93	2
86	Hungary	Farm16	RP		positive	32.14	3
87	Hungary	Farm16		N	negative		2
88	Hungary	Farm17	RP		positive	36.69	4
89	Hungary	Farm17	RP		positive	35.89	3
90	Hungary	Farm17	RP		positive	35.55	1
91	Hungary	Farm18	RP		negative		1
92	Hungary	Farm18	RP		negative		1
93	Hungary	Farm18		N	negative		1
94	Hungary	Farm18		N	negative		2
95	Hungary	Farm18		N	negative		2
96	Hungary	Farm18		N	negative		3
97	Hungary	Farm18	RP		negative		3
98	Hungary	Farm18		N	negative		1
99	Hungary	Farm19		N	positive	36.21	6
100	Hungary	Farm19		N	negative		2
101	Hungary	Farm19		N	positive	36.64	2
102	Hungary	Farm19	RP		positive	29.67	5
103	Hungary	Farm20		N	positive	35.64	1
104	Hungary	Farm20	RP		positive	27.08	4
105	Hungary	Farm20	RP		positive	33.64	1
106	Hungary	Farm20		N	negative		1
107	Hungary	Farm20		N	positive	35.43	2
108	Hungary	Farm20	RP		positive	33.07	3
109	Hungary	Farm20		N	positive	35.53	1
110	Hungary	Farm20		N	positive	34.46	1
111	Hungary	Farm20	RP		positive	35.01	5
112	Hungary	Farm20	RP		positive	33.23	4
113	Hungary	Farm20	RP		positive	30.28	1
114	Hungary	Farm20		N	positive	35.39	3
115	Hungary	Farm20		N	positive	35.71	2
116	Slovakia	Farm21		N	negative		2
117	Slovakia	Farm21	RP		positive	16.55	3
118	Hungary	Farm22		N	negative		1
119	Hungary	Farm22	RP		positive	33.06	3
120	Hungary	Farm23	RP		positive	14.72	8
121	Hungary	Farm23	RP		positive	32.93	4
122	Hungary	Farm23	RP		positive	32.33	5
123	Hungary	Farm23	RP		positive	32.68	1
124	Hungary	Farm23	RP		positive	34.44	1

125	Hungary	Farm23	RP		positive	32.84	3
126	Hungary	Farm24	RP		positive	35.51	3
127	Hungary	Farm25		N	positive	31.52	2
128	Hungary	Farm25	RP		positive	30.33	4
129	Hungary	Farm25	RP		negative		1
130	Hungary	Farm26		N	negative		2
131	Hungary	Farm26	RP		positive	27.07	4
132	Hungary	Farm26	RP		positive	35.05	1
133	Hungary	Farm26		N	negative		1
134	Hungary	Farm26	RP		positive	35.12	4
135	Hungary	Farm27	RP		positive	34.67	3
136	Slovakia	Farm28		N	negative		2
137	Slovakia	Farm28	RP		positive	34.34	6
138	Slovakia	Farm28		N	negative		2
139	Slovakia	Farm29		N	negative		2
140	Hungary	Farm30	RP		positive	11.92	3
141	Hungary	Farm30	RP		positive	33.33	4
142	Hungary	Farm30	RP		positive	34.11	3
143	Hungary	Farm30	RP		positive	28.61	4
144	Hungary	Farm30		N	positive	30.22	1
145	Hungary	Farm30	RP		positive	23.71	1
146	Hungary	Farm31	RP		positive	34.01	3
147	Hungary	Farm31	RP		positive	30.47	4
148	Hungary	Farm31		N	positive	34.11	2
149	Hungary	Farm31	RP		positive	23.29	3
150	Hungary	Farm31		N	positive	32.03	2
151	Hungary	Farm31	RP		positive	12.53	3
152	Hungary	Farm31		N	positive	34.25	2
153	Hungary	farm32	RP		positive	31.60	4
154	Hungary	Farm33		N	positive	35.82	2
155	Hungary	Farm33		N	positive	30.27	2
156	Hungary	Farm33	RP		positive	29.66	6
157	Hungary	Farm33	RP		positive	12.38	4
158	Hungary	Farm33		N	positive	33.85	2
159	Hungary	Farm33		N	positive	31.28	2
160	Hungary	Farm33	RP		positive	16.53	7
161	Hungary	Farm33		N	positive	28.43	2
162	Hungary	Farm33	RP		positive	17.80	5
163	Hungary	Farm34		N	positive	30.15	2
164	Hungary	Farm34	RP		positive	24.35	3
165	Hungary	Farm34		N	positive	31.91	4
166	Hungary	Farm34	RP		positive	29.02	3
167	Hungary	Farm35		N	negative		2

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