



AKADÉMIAI KIADÓ

Prevalence of *Coxiella burnetii* in bovine placentas in Hungary and Slovakia: Detection of a novel sequence type – Short communication

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SHORT COMMUNICATION



ABSTRACT

Cotyledons ($n = 167$) from 30 Hungarian and 5 Slovakian dairy cattle herds were analysed for *Coxiella burnetii* by real-time PCR targeting the *IS1111* gene. 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. Seventeen out of the 80 positive samples (21.3%) originating from retained placentas were found to be highly loaded with *C. burnetii* with a cycle threshold (Ct) value lower than or equal to 27.08, ranging between 11.92 and 27.08. The rest of the positive samples from retained fetal membranes and from normally separated placentas were moderately loaded with *C. burnetii* DNA. Five out of the ten samples showing the strongest positivity (Ct 11.92–18.28) from retained placentas were genotyped by multispacer sequence typing based on ten loci, which revealed sequence type (ST) 61, a type that had not been detected in Hungary and Slovakia previously. Retained placenta was more likely in cows with *C. burnetii* PCR-positive cotyledons (odds ratio: 12.61, $P = 0.0023$). The high *C. burnetii* DNA load found in retained fetal membranes may be a potential risk factor for human infection and may also be associated with the retention of fetal membranes.

KEYWORDS

Central Europe, *Coxiella burnetii*, dairy cattle, placenta, multispacer sequence typing, Q fever

Q fever is a zoonotic disease of worldwide distribution. The three most important reservoir host species of the aetiological agent, *Coxiella burnetii* are cattle, sheep and goats (Eldin et al., 2017). The placenta of infected animals contains the highest concentration of bacteria and it is the most important source of human infection. Cattle can shed *C. burnetii* also in the milk, faeces, and urine (Guatteo et al., 2007; Tissot-Dupont and Raoult, 2008). The pathogen initially infects the placenta and then it may spread to the fetus via the amniotic-oral or haematogenous route (Agerholm, 2013). The retention of fetal membranes is a common problem in periparturient dairy cows, but its exact aetiology has not been fully elucidated yet. Scientific evidence for *C. burnetii*-associated retained placenta was obtained in only one study involving a goat herd (Waldhalm et al., 1978).

Nowadays, there is an increased awareness of Q fever as an economically important disease on industrial farms. Infected animals mainly remain clinically healthy, but the presence of the bacteria may lead to economic losses through reduced fertility (Dobos et al., 2020a; Vourvidis et al., 2021).

The aims of the present study were to compare the occurrence of *C. burnetii* in retained fetal membranes and in normally separated placentas. A further objective was to identify the *C. burnetii* multispacer sequence typing (MST) genotypes occurring in 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 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

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veterinarians selected one cotyledon per placenta which was stored at -19°C on the farms. Cotyledons were sliced up and mixed with 10 mL of phosphate-buffered saline, then homogenised with a laboratory blender. The sediments of centrifuged homogenates were subjected to DNA extraction with a commercial kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). To quantify the approximate bacterial load in the samples, the cycle threshold (Ct) values of all samples were analysed by TaqMan type real-time polymerase chain reaction (PCR) assay targeting the multicopy *IS1111* insertion element of the *C. burnetii* genome (Loftis et al., 2006). The detection threshold of the PCR system was ~ 0.1 CFU (Ct 36.95), according to a commercially available positive control which was just used as a positive control (Adiavet Cox, Aes Chemunex Inc., Cranbury, NJ) (Table 1). For the MST analysis, ten selected spacer regions of the *C. burnetii* genome were amplified and sequenced as described by Glazunova et al. (2005). 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 used. Phylogeny was inferred by using the Neighbour-joining method and Tamura 3-parameter model estimated in MEGA X software (Tamura, 1992; Kumar et al., 2018).

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).

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 *IS1111* real-time PCR (Table 2). Seventeen (21.3%) out of these positive samples from retained placentas were highly loaded with *C. burnetii* with a Ct value not exceeding 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 (Table 2). 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 MST (Fig. 1). The newly determined sequences were deposited to GenBank and assigned to accession numbers MW441853–MW441902.

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 ($P = 0.0079$).

Recent studies have demonstrated that *C. burnetii* infection is highly prevalent on dairy cattle farms of the Central and Eastern European countries both at farm (93.78%) (Dobos et al., 2020b) and individual animal level (52%) (Dobos et al., 2020a). 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; Dobos et al., 2020a). 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

Table 1. Mean cycle threshold (Ct) values and equivalent estimated microorganism concentration (Colony Forming Unit (CFU)/ μL) thresholds of the PCR system compared to a commercially available positive control (Adiavet Cox, Aes Chemunex Inc., Cranbury, NJ)

CFU/ μL	Mean Ct value
1,000	23.68
100	27.08
10	30.82
1	33.60
0.1	36.95
0.01	negative

Table 2. Percentage of *Coxiella burnetii* positivity with different DNA loads in retained and normally separated placentas

	High DNA load (Ct \leq 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%)

Ct = cycle threshold.



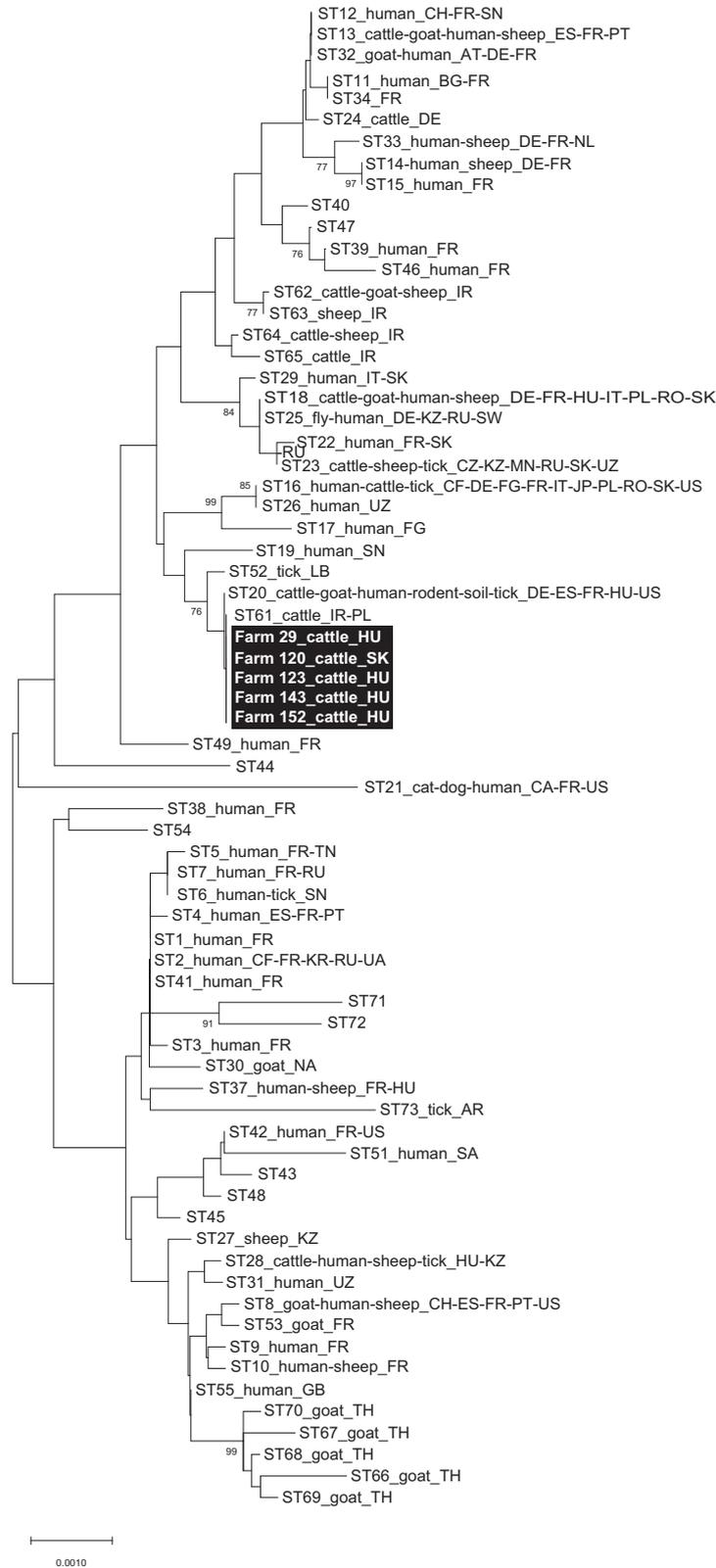
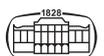


Fig. 1. Neighbour-joining tree showing the placement of the samples (highlighted area) from this study with known sequence types (STs). Bootstrap values of ≥ 70 are shown (1,000 replicates). The scale bar represents the average number of substitutions per site. Isolate origins and sources are given according 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)



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., 1952](#)), 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., 2020](#)), 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 ([Dobos and Balla, 2021](#)). 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, a microvariant (one nucleotide deletion) of ST20, which is the sequence type most often associated

with bovine samples and products globally and also in Hungary ([Santos et al., 2012](#); [Tilburg et al., 2012](#); [Sulyok et al., 2014](#); [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, but further studies are needed to determine the virulence and pathomechanism of these STs in cattle.

DECLARATION OF COMPETING INTERESTS

A. D. works for a company which is the marketing authorisation holder of a vaccine against the bacterium studied.

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