

1 **Rapid Communications**

2 **Outbreaks of abortions by *Coxiella burnetii* in small ruminant flocks and a longitudinal**
3 **serological approach on archived bulk tank milk suggest Q fever emergence in Central Portugal**

4 **Running title:** Q fever outbreaks and epidemiology in sheep of Portugal

5 Rita Cruz¹, Fernando Esteves¹, Carmen Vasconcelos-Nóbrega¹, Carla Santos¹, Ana S. Ferreira²,
6 Cristina Mega¹, Ana C. Coelho³, Helena Vala^{1,4}, João R. Mesquita^{1,5}

7 ¹Agrarian Superior School, Polytechnic Institute of Viseu, 3500-606 Viseu, Portugal

8 ²Laboratory of Microbiology, Department of Biological Sciences, Faculty of Pharmacy, University
9 of Oporto, 4050-313 Porto, Portugal

10 ³Animal and Veterinary Research Centre (CECAV), University of Trás-os-Montes and Alto Douro,
11 5001-801 Vila Real, Portugal

12 ⁴Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB),
13 University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal

14 ⁵Epidemiology Research Unit (EPIUnit), Institute of Public Health, University of Porto, 4050-313
15 Porto, Portugal

16 **Corresponding author:** JR Mesquita. Escola Superior Agrária de Viseu, Quinta da Alagoa. Estrada
17 de Nelas, Ranhados. 3500-606 Viseu. Portugal. Tlf: +351 232446600. Fax: +351 232426536. E-
18 mail address: jmesquita@esav.ipv.pt.

19 Cruz R: ritacruzpaiva@gmail.com | Esteves F: festeves@esav.ipv.pt | Vasconcelos-Nóbrega C:
20 cnobrega@esav.ipv.pt | Santos C: casarede@esav.ipv.pt | Ferreira AS:
21 anasofia.afonsoferreira@gmail.com | Mega C: amega@esav.ipv.pt | Coelho AC:
22 accoelho@utad.pt | Vala H: hvala@esav.ipv.pt | Mesquita JR: jmesquita@esav.ipv.pt

Summary

Q fever is a worldwide zoonotic infectious disease caused by *Coxiella burnetii* and sheep and goats are known to be the main reservoir for human infection. The present study describes the epidemiological and laboratory findings of *C. burnetii* outbreaks affecting sheep and goat flocks and also provides the results of a prospective serosurvey in bulk-tank milk samples to assess *C. burnetii* circulation in a population of sheep living in close contact to the human population in Central Portugal. In the epizooties, *C. burnetii* was identified in tissues of the resulting abortions by qPCR. As for the serological survey, 10.2% (95%CI: 4.5-19.2) of the 78 bulk tank milk samples collected in 2015 presented IgG antibodies against *C. burnetii*. The same farms were visited and sampled in 2016 and 25.6% (95%CI: 16.4-36.8) were positive. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant ($p = 0.020$) and is strongly suggestive of Q fever emergence in Central Portugal. Measures on animal health and on disease spread control to the human population should be considered.

Keywords: *Coxiella burnetii*, Q fever, small ruminants, outbreaks, epidemiology

Impacts

We report two outbreaks of Q fever in sheep and goat flocks

We describe a steep increase in Q fever antibodies in milk from sheep farms

There is the possibility for Q fever emergence in central Portugal

Introduction

Q fever is a worldwide zoonotic infectious disease caused by *Coxiella burnetii* and ruminants, namely, cattle, sheep and goats are known to be the main reservoir for human infection, however ticks are also considered a common reservoir (Arricau-Bouvery and Rodolakis, 2005; Van den Brom *et al.*, 2015; Angelakis and Raoult, 2010; Djerbib *et al.*, 2018). *C. burnetii* infection in ruminants can result in epizootic abortions, which are often associated with vast bacteria shedding in birth fluids and placentas, significantly increasing the risk of disease spread (O'Neill *et al.*, 2014; Filioussis *et al.*, 2017). Human infections mainly occur in persons handling infected animals and their products but until recently, the zoonotic transfer of *C. burnetii* to the human population did not generate important alerts in both Veterinary and Human Public Health (Tselentis *et al.*, 1995; Ergas *et al.*, 2006).

In the last decade, a strong paradigm shift has occurred in the scientific community due to a major epidemic that has occurred in the general population in the Netherlands, resulting in 3 525 notified cases in humans and the subsequent national cull of carrying goat herds (van der Hoek *et al.*, 2010). Abortion clusters in goat herds that started a few years earlier, as a consequence of the intensification of dairy goat production systems in the region, were initially suggested as the source of this large human epidemic (van der Hoek *et al.*, 2010). This was later supported by the results of geospatial studies indicating an association between the human cases and the dairy goat farms (Schimmer *et al.*, 2010). Since then, important work has been made to prevent the spread of *C. burnetii* from sheep or goats to humans, however these efforts have been hindered by the limitation of effective veterinary interventions in these small ruminants (Angelakis and Raoult, 2010).

Much is known on the role of sentinel animals in detecting risks to humans by providing early warning of an emerging infectious disease, being the particular case of sheep regarding *C. burnetii* (Mori *et al.*, 2018). In particular, the important tradition behind primary production of

sheep in the Centre of Portugal, mainly done at a small scale and in intimate contact with humans, can provide seroepidemiological studies with sentinel flocks and thus potentially mitigate *C. burnetii* shedding to the human population. The present study describes two epizooties of *C. burnetii* affecting sheep and goat flocks, and also provides the results of a 2-year prospective serosurvey in bulk-tank milk samples to assess *C. burnetii* circulation in a population of sheep living in close contact to the human population in Central Portugal.

Materials and methods

Outbreaks investigation

The first outbreak of abortions started on 15 november 2017 and lasted for 2 months in a sheep farm in the municipality of Mangualde (40.58633 North; -7.760661 West), district of Viseu, Central Portugal. The flock was not vaccinated to Q fever (only vaccinated against brucellosis) and had sheep from “Serra da Estrela” breed, the autochthonous breed of this region that produces the best sheep milk in Portugal. This milk is used for the highly valued and recognized worldwide, cheese “Serra da Estrela”. There were 155 sheep in the flock, of which 100 were pregnant and 20 aborted. The first abortion occurred in 15 November 2017 and the last in 18 January 2018. Aborted fetuses (n=2) were taken and refrigerated until arriving at the Laboratory (within 24 h). The second outbreak of abortions started on 10 January 2018 and lasted for 3 weeks in a goat farm in the municipality of Aguiar da Beira (40.81443 North; -7.54440 West), also in the district of Viseu, Central Portugal, approximately 50 km distant from the first outbreak. The flock had goats from “Murciana” breed and was also not vaccinated to Q fever (only against brucellosis). There were 60 goats in the flock, all pregnant, of which 25 aborted. The first abortion occurred in 10 January 2018 and the last in 30 January 2018. Placenta (n=1) was taken and refrigerated until arriving at the Laboratory (within 24 h). Tissues from both outbreaks were tested for a panel of abortion pathogens, namely *Toxoplasma gondii*,

Chlamydiaceae and *Coxiella burnetii*. DNA was extracted using NucleoSpin® Tissue kit, (Macherey Nagel, Duren, Germany), according to the manufacturers instructions. For pathogen genomic detection, 3 commercial real-time PCR probe assay kits with endogenous controls were used, according to the manufacturers instructions (EXOone *Toxoplasma gondii* oneMIX Kit; EXOone *Chlamydiaceae* one MIX Kit; EXOone *Coxiella burnetii* oneMIX Kit, Zaragoza, Spain). All reactions were performed using a positive, a negative and an endogenous control (β -actin target).

A questionnaire was applied to the farm owners and families, as well as to the workers for symptoms fitting acute Q fever case definition (acute fever and one or more of the following: rigors, severe retrobulbar headache, acute hepatitis, pneumonia,) (CDC, 2009), during the period of the outbreaks and the following months, with the intervention and help of a human health team (a medical doctor and a nurse) and none fit the case definition.

To address *C. burnetii* spread the delivered questionnaires included queries on similar epizootic abortions in nearby farms during the period of the outbreaks and the following months. The farm owner from the first outbreak reported that a neighbouring sheep farm had experienced 5 to 7 abortions during the same period. Despite efforts we were not able to retrieve epidemiological data and samples from that sheep farm for analysis.

Bulk tank milk collection

The study geographical location was Estrela Mountain (“Serra da Estrela”), located in central Portugal, where the National Association of Serra da Estrela Sheep breed - ANCOSE (Associação Nacional de Criadores de Ovinos da Serra da Estrela; <http://www.ancose.com>) is responsible for the administration of one of Portugal’s autochthonous sheep breeds, the “Serra da Estrela”. This breed provides for several European Union’s Protected Designation of Origin (PDO) products, being exclusively bred in this region and thus having residual animal movement due to its confined production to the farm premises. For the serological analysis to study *C. burnetii*

emergence, samples from a previous study on Schmallenberg virus (data not published) were used. All registered sheep flocks (ANCOSE official, N=180) were invited to participate in this study, which required a bulk tank milk collection (one in January/February 2015, the other in January/February 2016). A total of 78 sheep milk farms from 46 parishes of 5 municipalities the Centre region of Portugal (Celorico da Beira, Fornos de Algodres, Gouveia, Seia and Tábua) answered and accepted to participate (response rate= 43.3%). The farm where the 2017 abortion outbreak occurred did not participate. All farms provided a 2 ml bulk-milk sample both in January 2015 and in January 2016, which was swiftly transported to the laboratory at 4 °C. Samples were processed according to the manufacturer's instructions with slight modifications (Chaintoutis *et al.*, 2014). Briefly, bulk-milk samples were centrifuged at 1,000×g at 4 °C for 10 min. After centrifugation, the fat fraction was removed using a sterile spatula, and the remaining fraction was transferred to a 1.5 ml microcentrifuge tube and immediately frozen (-20 °C) until analysis.

Enzyme linked immunosorbent assay

Samples were tested for the presence of anti-*C. burnetii* IgG antibodies, using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), following the manufacturer's instructions. The positive control of this assay is a pool of positive bovine sera (field infected, from France) and the assay has a sensitivity and a specificity of 100% (according to the manufacturer). For test interpretation, sample-to-positive control (S/P) ratio in each serum was calculated, according to the formula provided: $S/P = (OD_{450} \text{ sample} - OD_{450} \text{ NC}) / (OD_{450} \text{ PC} - OD_{450} \text{ NC})$; where $OD_{450} \text{ sample}$ = optical density of the sample, $OD_{450} \text{ NC}$ = optical density of the negative control and $OD_{450} \text{ PC}$ = optical density of the positive control. Results were expressed as an index (S/P x 100). Indices stratified as 3 different rising categories. Samples with S/P indices <30% were considered negative, samples with S/P indices between 30 and 40% were considered doubtful, samples with S/P indices >40% were considered positive. Doubtful samples were retested and if resulting doubtful, considered as negative. Obtained data

were used to calculate NUTS II-specific seroprevalence values and differences between NUTS II-specific seroprevalence, in 2015 and 2016, were evaluated by Fisher's exact test, using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and considered statistically significant if $p < 0.05$. Exact binomial 95% confidence intervals (CI) were established for proportions.

Results and discussion

Results from the qPCR screening in the aborted fetuses and placenta showed that samples were negative for *T. gondii* and Chlamydia, and positive for *C. burnetii*, strongly suggesting that the abortion outbreaks that affected both farms were due to Q fever. Moreover, both flocks are vaccinated against brucellosis, thus excluding also this pathogen from the algorithm for epidemic abortions. All farm owners, family members and workers replied to the questionnaire however none fitted acute Q fever case definition. Nonetheless, the genotypes involved in the outbreaks could have been associated to low virulence in humans, and asymptomatic infections could have occurred (Van Schaik *et al.*, 2013)

As for the serological survey, anti-*C. burnetii* antibodies were found in both years. From the 2015 sampling, eight (10.2%; 95%CI: 4.5-19.2) of the 78 bulk tank milk samples presented IgG antibodies against *C. burnetii*, while from the 2016 sampling, 20 (25.6%; 95%CI: 16.4-36.8) of the total 78 bulk tank milk samples were positive. Of the eight initially (2015) positive farms, five (62.5%) maintained their seropositive status regarding Q fever and three (37.5%) became seronegative. Of the anti-*C. burnetii* seronegative farms from 2015, 15 (25.8%) had seroconverted by 2016. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant ($p = 0.020$).

Regarding the distribution of IgG anti-*C. burnetii* positive bulk tank milk samples, according to geographical location (Table 1), in 2015 and 2016, an increase was observed in all but one municipality, namely in Seia (8.7% [CI: 1.1-28.0%] versus 21.7% [CI: 7.5-43.7%]), Gouveia (18.8%

[CI: 4.0-45.6%] versus 31.3% [CI: 11.0-58.7%]), Celorico da Beira (0% [CI: 0.0-0.0%] versus 26.7% [CI: 7.8-55.1%]) and Tábua (12.5% [CI: 1.6-38.3%] versus 31.3% [CI: 11.1-58.7%]). Only Fornos de Algodres showed no change in the number of IgG anti-*C. burnetii* positive bulk tank milk samples (12.5% [CI: 0.3-52.7%]). The steep increase of IgG anti-*C. burnetii* bulk tank milk samples across the Central region of Portugal is strongly suggestive of Q fever emergence in Central Portugal.

ELISA on bulk tank milk samples have shown in the past to be a valuable matrix for the screening of *C. burnetii* infection within animals in lactation by providing information about the exposure to *C. burnetii* (Guatteo *et al.*, 2007, van den Brom *et al.*, 2012) and producing comparable results to those obtained in serum samples due to the immunoglobulin transfer from blood to milk (Nielsen *et al.*, 2011). Interestingly another study in Portugal has detected IgG anti-*C. burnetii* in milk (Anastácio *et al.*, 2016). Authors have collected 39 bulk milk samples from sheep in the same region, from 2009 to 2013, and showed that 51.3% flocks had positive samples (Anastácio *et al.*, 2016). Although authors have obtained a much higher prevalence of positive bulk tank milk samples, they have tested samples for the presence of specific anti-*C. burnetii* antibodies using a different commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever; Laboratoire Service International, Lissieu, France). Thus we find difficult to compare values as a different assay with distinct sensitivity/specificity has been used in the present study.

While considered to remain unaltered, Q fever prevalence in the human population of Portugal is conflicting with European data that shows a clear increase of cases (DGS, 2015; ECDC, 2014). Although little is known regarding Q fever in Portugal, a few recent case reports in humans have been linked to animals, highlighting the concern for zoonotic transfer from ruminants (Alves *et al.*, 2016). It is therefore of the upmost importance to provide results on the circulation of *C. burnetii* in sheep, so as to implement measures on animal health and control the disease spread to the human population.

Acknowledgments

This work is financed by national funds through FCT - Fundação para a Ciência e Tecnologia, I.P., under the projects UID/Multi/04016/2016, FCOMP-01-0124-FEDER-009525, UID/AGR/04033/2013 and SBVEPS (Procº 441.00 SERVIA); QREN/FEDER under the project Ovislab ICT-2013-05-004-5314 ID-64757; CI&DETS and CGD under projects rumDISEASE PROJ/CI&DETS/2016/0023, SBMERGE PROJ/CI&DETS/CGD/009 and HEALTHY-ValorWhey PROJ/CI&DETS/CGD/007); and FEDER/COMPETE/POCI under project POCI-01-0145-FEDER-006958. We would like to thank UTAD, CITAB and 3rd year CTA for their support.

Conflict of interest

Authors have no conflict of interest

References

1. Alves, J., Almeida, F., Duro, R., Ferraz, R., Silva, S., Sobrinho-Simões, J., & Sarmento, A. (2016). Presentation and diagnosis of acute Q fever in Portugal - A case series. *IDCases*, 7, 34-37. doi: 10.1016/j.idcr.2016.11.002
2. Anastácio, S., Carolino, N., Sidi-Boumedine, K., & da Silva, G. J. (2016). Q Fever Dairy Herd Status Determination Based on Serological and Molecular Analysis of Bulk Tank Milk. *Transboundary and Emerging Diseases*, 63, e293-300. doi: 10.1111/tbed.12275
3. Angelakis, E., & Raoult, D. (2010). Q Fever. *Veterinary Microbiology*, 27, 297-309. doi:10.1016/j.vetmic.2009.07.016
4. Arricau-Bouvery, N., & Rodolakis, A. (2005). Is Q fever an emerging or re-emerging zoonosis? *Veterinary Research*, 36, 327-49.
5. CDC. (2009). Q Fever (*Coxiella burnetii*) 2009 Case Definition. (available: <https://wwwn.cdc.gov/nndss/conditions/q-fever/case-definition/2009/>)
6. Chaintoutis, S.C., Kiossis, E., Giadinis, N.D., Brozos, C.N., Sailleau, C., Viarouge, C., Bréard, E., Papanastassopoulou, M., Zientara, S., Papadopoulos, O., & Dovas, C.I. (2014). Evidence of Schmallenberg virus circulation in ruminants in Greece. *Tropical Animal Health Production*, 46, 251-5. doi:10.1007/s11250-013-0449-5
7. DGS. (2015). Direção-Geral da Saúde. Doenças de Notificação Obrigatória (1999 a 2013). Portugal.
8. Djerbib, A., Czaplicki, G., Grégoire, F., Kirschvink, N., Saegerman, C., & Dal Pozzo, F. (2018). Exploratory investigation of Q fever in apparently healthy meat sheep flocks in Belgium. *Transboundary and Emerging Diseases*. e-pub. doi: 10.1111/tbed.12850.

9. ECDC. (2014). Surveillance Report Annual epidemiological report–Emerging and vector-borne diseases.
10. Ergas, D., Keysari, A., Edelstein, V., & Sthoeger, Z. M. (2006). Acute Q fever in Israel: clinical and laboratory study of 100 hospitalized patients. The Israel Medical Association Journal, 8, 337-41.
11. Filioussis, G., Theodoridis, A., Papadopoulos, D., Gelasakis, A. I., Vouraki, S., Bramis, G., & Arsenos, G. (2017). Serological prevalence of *Coxiella burnetii* in dairy goats and ewes diagnosed with adverse pregnancy outcomes in Greece. Annals of Agricultural and Environmental Medicine, 23, 702-705. doi:10.26444/aaem/80706
12. Guatteo, R., Beaudreau, F., Joly, A., & Seegers, H. (2007). Assessing the within-herd prevalence of *Coxiella burnetii* milk-shedder cows using a real-time PCR applied to bulk tank milk. Zoonoses Public Health, 54, 191-4. doi:10.1111/j.1863-2378.2007.01043.x
13. Mori, M., & Roest, H. J. (2018). Farming, Q fever and public health: agricultural practices and beyond. Archives Public Health, 6, 76:2.
14. Nielsen, K. T., Nielsen, S. S., Agger, J. F., Christoffersen, A. B., & Agerholm, J. S. (2011) Association between antibodies to *Coxiella burnetii* in bulk tank milk and perinatal mortality of Danish dairy calves. Acta Veterinaria Scandinavica, 2, 53:64.
15. O'Neill, T. J., Sargeant, J. M., & Poljak, Z. (2014). A systematic review and meta-analysis of phase I inactivated vaccines to reduce shedding of *Coxiella burnetii* from sheep and goats from routes of public health importance. Zoonoses Public Health, 61, 519-33.
16. Schimmer, B., Ter Schegget, R., Wegdam, M., Züchner, L., de Bruin, A., Schneeberger, P.M., Veenstra, T., Vellema, P., & van der Hoek W. (2010). The use of a geographic information system to identify a dairy goat farm as the most likely source of an urban Q-fever outbreak. BMC Infectious Diseases. 16, 69. doi:10.1186/1471-2334-10-69

- 254 17. Tselentis, Y., Gikas, A., Kofteridis, D., Kyriakakis, E., Lydataki, N., Bouros, D., & Tsaparas,
255 N. (1995). Q fever in the Greek Island of Crete: epidemiologic, clinical, and therapeutic
256 data from 98 cases. *Clinical Infectious Diseases*, 20, 1311-6.
- 257 18. Van Schaik, E.J., Chen, C., Mertens, K., Weber, M.M., & Samuel, J.E. (2013) Molecular
258 pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. *Nature reviews*
259 *Microbiology*, 11,561-573.
- 260 19. van den Brom, R., van Engelen, E., Luttikholt, S., Moll, L., van Maanen, K., & Vellema, P.
261 (2012). *Coxiella burnetii* in bulk tank milk samples from dairy goat and dairy sheep farms
262 in The Netherlands in 2008. *Veterinary Record*, 24, 310.
- 263 20. van der Hoek, W., Dijkstra, F., Schimmer, B., Schneeberger, P. M., Vellema, P., &
264 Wijkmans, C. (2010). Q fever in the Netherlands: an update on the epidemiology and
265 control measures. *Euro Surveillance*, 15, pii:19520.

Table 1. Screening for anti-*C. burnetii* IgG antibodies in bulk-milk tank samples from Serra da Estrela sheep, years 2015 and 2016

Municipality	2015	2016	P value
	SBV IgG positive/Total: no. (%; CI)	SBV IgG positives/Total: no. (%; CI)	
Seia	2/23 (8.7; 1.1-28.0)	5/23 (21.7;7.5-43.7)	0.414
Gouveia	3/16 (18.8;4.0-45.6)	5/16 (31.3;11.0-58.7)	0.685
Fornos de Algodres	1/8 (12.5;0.3-52.7)	1/8 (12.5;0.3-52.7)	†
Celorico da Beira	0/15 (0.0;0.0-0.0)	4/15 (26.7;7.8-55.1)	0.099
Tábua	2/16 (12.5; 1.6-38.3)	5/16 (31.3; 11.1-58.7)	0.394
Total	8/78 (10.2; 4.5-19.2)	20/78 (25.8; 16.4-36.8)	0.021 *

CI: 95% confidence interval; †: not determined; * p value < 0.05