Spanish Journal of Agricultural Research
16 (2), e05SC01, 6 pages (2018)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2018162-12180
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A, M.P. (INIA)

SHORT COMMUNICATION

OPEN ACCESS

Broadening the diagnosis panel of reproductive pathogens associated with abortion in ruminants

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Abstract

Diagnosis of abortion in cattle, sheep and goat have been mainly focused on abortive pathogens with a recognized impact in outbreaks, but the aetiologic diagnosis rates have been historically low worldwide. Thus, we analysed the presence of pathogens in abortion outbreaks, focusing on the less-common pathogens in cattle farms with control programmes for reproductive pathogens, and in ovine and caprine farms. Thirty-one cases from Galician farms submitted to our laboratory during 2013-2015 were analysed (16 bovine, 7 ovine and 8 caprine farms) by polymerase chain reaction and culture from foetal tissues (n = 52 foetuses). Diagnosis was reached in 21/31 farms: 9/16 in bovine, 6/7 in ovine and 6/8 in caprine. *Campylobacter* spp. were found in all three species (3/9 diagnosed cases in bovine, 2/6 in ovine and 4/6 in caprine). Furthermore, *Ureaplasma diversum* was detected in cattle (4/9 of diagnosed cases), Bovine Viral Diarrhoea Virus – 2 was detected in sheep (2/6) and *Neospora caninum* in goats (1/6). Our results prove the occurrence of abortion in response to pathogens that are traditionally considered less relevant and rarely included in the diagnosis of ruminant abortion. Therefore, differential diagnosis of abortion should consider these pathogens (at least when other causes have been ruled out), to effectively control abortion in farms.

Additional keywords: cattle; sheep; goat; PCR; Spain .

Abbreviations used: ADSG (Health Defense Group, *Agrupación de Defensa Sanitaria Ganadera*); BoHV (Bovine Herpesvirus); BVD (Bovine Viral Diarrhoea); BVDV (Bovine Viral Diarrhoea Virus); IBR (Infectious Bovine Rhinotracheitis); PCR (Polymerase Chain Reaction).

Authors' contributions: Conceived and designed the experiments and interpretation of data: JMD and GF. Performed the experiments: JMD and AP. Wrote the paper: JMD. All the authors made a critical revision of the manuscript for important intellectual content and approved the final version.

Citation: Díaz-Cao, J. M.; Prieto, A.; López-Lorenzo, G.; Díaz-Fernández, P.; López-Sández, C.; Morrondo, P.; Fernández-Rodríguez, G. (2018). Short communication: Broadening the diagnosis panel of reproductive pathogens associated with abortion in ruminants. Spanish Journal of Agricultural Research, Volume 16, Issue 2, e05SC01. https://doi.org/10.5424/sjar/2018162-12180

Received: 03 Sept 2017. **Accepted:** 12 Jun 2018.

Funding: Xunta de Galicia, Spain, Programme for consolidating and structuring competitive research groups (GRC2015/003); Xunta de Galicia (predoctoral grant to JMDC).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Diagnostic rates in ruminant abortions are low worldwide, reaching approximately 50% of the cases (Anderson, 2007; Moeller, 2012; Matthews, 2016). Most diagnostic laboratories use a standardized diagnostic protocol consisting of a panel of tests (Anderson, 2007) that generally include pathogens that are considered important in abortion outbreaks, such as Bovine Viral Diarrhoea (BVD) viruses type 1 (BVDV-1) and type 2 (BVDV-2), Bovine Herpesvirus-1 (BoHV-1), *Neospora caninum* and *Leptospira* spp. in cattle, and *Chlamydia abortus* and *Toxoplasma gondii* in small ruminants. Other pathogens such as *Campylobacter* spp., *Salmonella* spp., or *Listeria* spp. are considered occasional (Borel *et al.*, 2014), so their presence in panels of diagnosis is variable and it depends on the laboratory.

Additionally, control programmes carried out by Health Defence Groups (ADSG, *Agrupación de Defensa Sanitaria Ganadera*) in cattle usually include some of the most important reproductive pathogens (BVDV, BoHV-1 and *N. caninum*). However, abortions are frequently an important problem on these farms and the relative situation to the presence of other pathogens potentially involved in reproductive failure is often unknown.

In this context, a narrow range of pathogens tested or controlled may contribute to the low efficiency in the diagnosis. To avoid this, in addition to the

aforementioned important pathogens for each species, we have included in the differential diagnosis pathogens variably considered by laboratories such as *Campylobacter* spp., *Salmonella* spp., *Coxiella burnetii* or *Listeria* spp. and pathogens rarely included in panels of diagnosis in Spain, such as *U. diversum* in cattle and *N. caninum* and *Leptospira* spp. in small ruminants. This panel was applied to a group of bovine, ovine and caprine farms within ADSG and suffering of abortion outbreaks, in spite of the existence of control programmes. Our aim was to identify the presence of pathogens that are not frequently investigated or present in control programmes in abortion outbreaks in farms with ongoing control programmes for some reproductive pathogens.

Material and methods

Study design

The study was carried out in the region of Galicia (NW Spain), which accounts for the highest census in dairy cattle (351,625 milking cows) in Spain, and a moderate-to-low population of small ruminants (172,486 sheep and 54,381 goats) (MAPAMA, 2016).

Cases of abortion included in this study consisted of abortion outbreaks submitted to our laboratory for diagnosis during 2013-2015. The participation in the study was voluntary and offered to the farms enrolled in five cattle ADSGs and in the single small ruminant ADSG in this region. These farms have carried out surveillance programmes consisting of laboratorial diagnosis in case of abortion outbreaks for some pathogens [BVD, IBR (Infectious Bovine Rhinotracheitis) and *N. caninum* in cattle and *T. gondii* and *C. abortus* in small ruminants]. Additionally, cattle farms performed control programmes for BVD, BoHV-1 and *N. caninum* during the prior five years by taking periodic samples to determine their sanitary status. Forty-two farms reported abortions with no cause determined by the control programmes. Out from them, to be included in this study, farms had to present abortion outbreaks, *i.e.* an annual abortion rate of > 5% (Menzies, 2011; Holler, 2012), were selected. As most farms in Galicia are small or medium-sized (Consellería do Medio Rural, 2015), bovine farms had also to present at least two abortions in the prior month. In ovine and caprine farms an outbreak was also included when a clustering of more than five abortions occurred within a 3-week period.

A case of abortion was defined as the set of samples from a same farm with an abortion outbreak and its duration was considered the number of months since farmer detection to the time of sample submission. Samples submitted could consist of foetal tissues, vaginal swabs or sera (up to 10 sera from aborted animals and up to 10 from healthy animals). Samples from the same farm were considered valid for diagnosis when at least two of the aforementioned sample types were submitted, being foetal tissues one of them.

Laboratory analysis

Foetal samples and vaginal swab samples were analysed using commercial polymerase chain reaction (PCR) kits. The diagnostic panel included all principal pathogens in ruminant abortions, as well as those not routinely investigated in laboratories, as detailed above: BoHV-1, BVDV-1/BVDV-2, Border Disease Virus (BDV), *Campylobacter* spp., *C. burnetii, N. caninum, Leptospira* spp. *Salmonella enterica* spp., *T. gondii* (LSI VetMAX™, Thermo-Fisher Scientific, Waltham, MA, USA), *U. diversum*, (Uredivdtec, GPS, Elche, Spain) and *C. abortus* (EXOPOL, Zaragoza, Spain). BVDV-1/BVDV-2 and BDV were initially screened using a PCR that detects the three pestiviruses (LSI VetMAX™, Thermo-Fisher Scientific, Waltham, MA, USA). Positive samples were further analysed by PCR to identify the specific species (BVDV Screening genesig®, Primerdesign™, Chandler's Ford, UK). Target tissues and pathogens analysed in each species are shown in Table 1. *Brucella* spp. were not considered since this infection is part of a compulsory official control programme and Galicia is declared free of caprine and ovine brucellosis and the herd prevalence of bovine brucellosis is declared to be 0% (MAPAMA, 2018).

Nucleic acid extraction was performed using the commercial kits Nucleospin Tissue and Nucleospin RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The initial material for extraction was 25 mg for brain and 200 µL for abomasal content. 0.1 g of liver, spleen, kidney and lung were pooled and macerated in 16 mL of PBS in a homogenizer (Stomacher®, Seward, Worthing, UK), and 200 µL of the homogenate used for DNA/RNA extractions. Vaginal swabs were eluted in 1 mL of PBS using 200 µl of the eluate for the extraction. PCRs were run in an ABIPRISM 7500 thermocycler (Thermo-Fisher Scientific, Waltham, MA, USA).

Bacteriology was used to detect other bacterial agents potentially related to abortions. Two blood agar plates were inoculated with 30 µL of abomasal content and incubated at 37ºC for 72 h. Daily readings were performed and colonies were identified by Gram-staining and biochemical tests.

Table 1. Results from PCR and bacteriology. Positive samples are indicated in parenthesis.

Sample	Pathogen	Number of diagnosed cases[a]		
		Cattle	Sheep	Goats
Microbiological culture				
Foetal tissues: Abomasal content	Total bacterial pathogens	3 (4)	0	1 (1)
	Aeromonas caviae + *Trueperella pyogenes*	1 (1)	0	0
	Trueperella pyogenes + *Streptococcus spp*	2 (2)	0	0
	Streptococcus spp.	1 (1)	0	0
	Listeria spp.	0	0	1 (1)
PCR				
Foetal tissues:				
Organ pools (liver, spleen, kidney and lung)	*Leptospira* spp.	0	1 (2)	0
	IBR	0		
	Total pestivirus: BVDV/BDV	0	2 (4)	0
	BVDV1	1(1)	0	0
	BVDV2	0	2 (4)	0
	BDV	0	0	0
	Ureaplasma diversum	4 (6)		
	Salmonella enterica spp.	0	0	0
Brain	*Neospora caninum*	1[b] (1)	0	1[c] (1)
	N. caninum + *Campylobacter spp.*	1 (1)	0	0
	Toxoplasma gondii		0	0
Abomasal content	*Campylobacter* spp.	3 (5)	2 (4)	4 (6)
Cases/foetus with a diagnosis by culture and PCR		9[d]/17	5/10	5[e]/8
Total cases/foetus analysed by culture and PCR		16/25	7/11	8/16
PCR				
Vaginal swabs from aborted animals	*Chlamydia abortus*	0	1 (2)	1 (2)
	Coxiella burnetii[f]	0	0	0
Cases/vaginal swabs with a diagnosis by PCR		0/0	1/2	1/2
Total cases/vaginal swab analysed by PCR		16/25	7/11	8/16

[a]A case of abortion is composed of various samples. [b]Sera results: 9/10 positive aborted; 3/10 positive unaborted ($p = 0.019$) *Campylobacter* spp. [c]Sera results: 6/6 positive aborted; 2/10 positive unaborted ($p = 0.007$). [d]In four cases, mixed diagnosis: (1) *A. caviae* + *T. pyogenes* and *T. pyogenes* + *Streptococcus* spp. (2) *T. pyogenes* + *Streptococcus* spp. and *Streptococcus* spp. (3) *U. diversum* and *Campylobacter* spp. (4) *N. caninum* + *Campylobacter* spp. [e]In one case a mixed diagnosis: (1) *Listeria* spp. and *Campylobacter* spp. [f]No sera analysis were performed because the pathogen was not detected by PCR.

Serological analyses were only performed if DNA of *N. caninum* and *C. burnetii* was detected by PCR. Commercial *N. caninum* Indirect Multi-species (IDVET, Montpellier, France) and LSIVet Ruminant Q Fever (Thermo-Fisher Scientific, Waltham, MA, USA) ELISA kits were used to detect specific antibodies.

Diagnosis criteria

Abortion of bacterial origin was diagnosed when bacteria were isolated in pure or nearly pure culture from foetal tissues or abomasal content, or when DNA of the considered pathogens was detected in foetal tissues by PCR, with two exceptions. First, *C. abortus* was considered the cause of abortion when it was detected in vaginal swabs (Güler *et al.*, 2006). Second, *C. burnetii* was considered the aetiological agent if detected in foetal samples or vaginal swabs in two aborted animals, or if only one aborted animal tested positive by PCR but more than 50% of sera samples from aborted animals tested positive by ELISA (Sidi-Boumedine *et al.*, 2010).

Viral diagnoses were made by detecting viral DNA/RNA in foetal tissues by PCR.

N. caninum or *T. gondii*-induced abortion was determined when DNA from these microorganisms was found in foetal tissues. A *N. caninum* diagnosis was only reached after the percentage of seropositive

animals was confirmed to be significantly higher in aborted animals than in unaborted animals by Fisher's exact test.

Abortion was considered undetermined when none of the considered pathogens were detected or fulfilled the diagnostic criteria.

Results and discussion

Thirty-one of 42 available farms were defined as cases of abortion outbreaks and included in this analysis. There were 16 bovine, 7 ovine and 8 caprine farms and a total of 52 analysed foetuses: 25 bovine, 11 ovine and 16 caprine. Compatible causes for abortion were identified in 21/31 farms: 9/16 bovine, 6/7 ovine and 6/8 caprine (Figure 1). The results are broken down by pathogen and sample type in Table 1. Overall, these results prove the presence of atypical pathogens in ruminant abortions, highlighting the diagnosis of *U. diversum* in 4/16 bovine farms, BVDV-2 in 2/7 ovine farms and *Campylobacter* spp. in 3/16 bovine farms, 2/7 ovine farms and 4/8 caprine farms. In cattle, both *U. diversum* and *Campylobacter* spp. have been considered a sporadic abortifacient (Anderson, 2007), but they may also constitute one of the most frequently diagnosed causes in a region (Campero *et al.*, 2003; Syrjälä *et al.*, 2007). Speciation of *Campylobacter* spp. was not performed, but since all bovine herds performed artificial insemination and there is no evidence of venereal transmission of *Campylobacter* spp. in small ruminants (Timoney *et al.*, 1988), faecal origin is the most likely source of infection. In small ruminants, some of the most important abortifacients in ovine/caprine were not extensively identified (*T. gondii, C. abortus*). In contrast, *Campylobacter* spp., *Leptospira* spp., *Listeria* spp. and *N. caninum* were found herein.

Twenty farms with a diagnosis provided information regarding the duration of problems. An apparent difference between the three species was observed. The six ovine diagnosed cases were all short-term outbreaks with less than 1 month of duration, which is consistent with lambing management in this species. In bovine, a large duration of problems was the most frequent situation (4/8 > 6 months; 3/8 between 1-6 months; 1/8 < 1 month), indicating persistent herd complications. In caprine the duration of problems was < 1 month in 2/6, 1/6 between 1-6 months and 3/6 more than 6 months, which may indicate episodes of persistence between lambing seasons.

The incidence of abortion is not consistently documented in this region. After consulting practitioners, mean and median values of annual percentage of abortions in cattle could be estimated around 7.59% and 4.9% In small ruminants, around 10% of the farms enrolled in the ADSG would submit samples

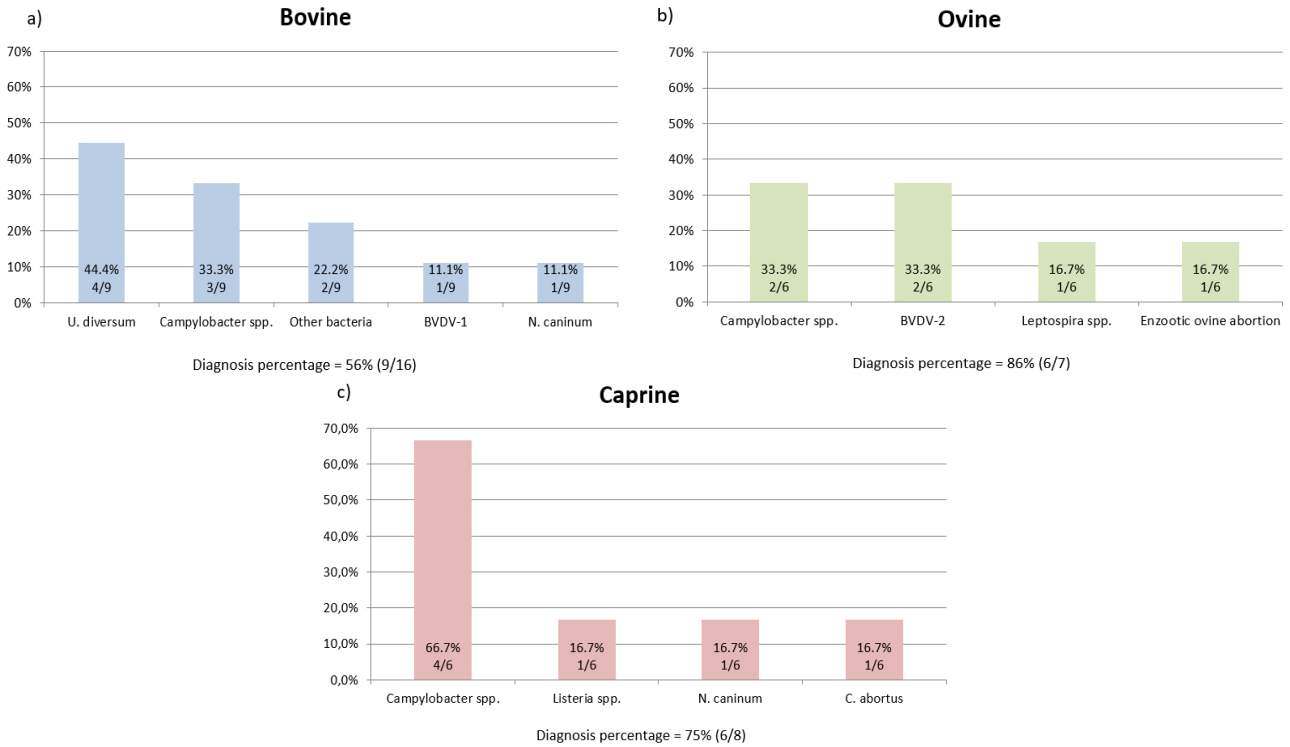

Figure 1. Frequency of diagnosed pathogens in each species (bovine (a), ovine (b) and caprine (c)) regarding to the total diagnosed cases and percentage of diagnosis regarding to the total cases. Some cases had a mixed diagnosis.

to the laboratory for abortion diagnosis yearly. The identification of an aetiologic agent is not achieved in about 55-70% of the cases, according to practitioners in the zone. The prevalence of pathogens in this region is not extensively reported. A study underlined *C. burnetii* in 9/44 cattle abortions, but the diagnosis was only achieved in the 34.1% of cases (González-Warleta *et al.*, 2016). This situation was not found in our study; however the differences may due to the low sample sizes or the different methodologies used in both studies. The exposure to some reproductive pathogens has been reported. Animal seroprevalences of 25% for BVD (Eiras, 2010), 38.4-35.7% for IBR (Eiras, 2010) and 15.7-24.1% for *N. caninum* (González-Warleta *et al.*, 2008; Eiras, 2010; Panadero *et al.*, 2010) are documented in cattle. In small ruminants, animal seroprevalences for *T. gondii* were 38.1-57% in sheep (Panadero *et al.*, 2010; Díaz *et al.*, 2014) and 48% in goats (Díaz *et al.*, 2016); and for *N. caninum*, 5.5-10.1% in sheep (Panadero *et al.*, 2010; Diaz *et al.*, 2014) and 6% in goats (Diaz *et al.*, 2016). In sheep, a serological study showed high seropositivity to *T. gondii* (38.1%) and low for *N. caninum, C. burnetii, C. abortus* and Pestivirus (< 8%) with only 1/44 farm with problems of abortion (Diaz *et al.*, 2014). Prevalence studies for other pathogens are sparse and focused on limited areas (Díaz Cao, 2016).

Our study offers some information regarding the problematic of abortions in this region. However, it should be noted that due to the sampling method and the low number of cases analysed, this study cannot aim to be representative of the prevalence of abortive agents. Actually, it could be expected that the control programmes for some pathogens could increase the relative importance of other infections, as it has been shown to occur in bovine mastitis (Fernandez *et al.*, 2013). Nevertheless, our results prove that other pathogens not routinely considered in the differential diagnosis of abortions may be present in these farms and consequently, they should be considered in the differential diagnosis of abortion. The addition of *U. diversum*, as well as *N. caninum* and *Leptospira* spp. in small ruminants, and *Campylobacter* spp. in the three species increased our aetiologic diagnostic rates from 4/16 (25%) to 9/16 (56.25%) in bovine, from 3/7 (42.86%) to 6/7 (85.71%) in ovine and from 2/8 (25%) to 7/8 (87.5%) in goats. Low sample sizes do not make these percentages very reliable, but they indicate that some gain in the diagnostic rates may be obtained by adding some pathogens to the diagnosis.

The low number of cases submitted to our laboratory could have been due to the costs of analysing a wide panel of pathogens. In addition, low rates of diagnosis of abortions can make it economically unattractive. However, narrow-range diagnostic panels can fail to make a diagnosis with subsequent negative economic consequences for farms. We recommend the inclusion of these commonly excluded pathogens as a second step, after discarding those others of recognized importance. In summary, our results emphasize the importance of continuing research to reveal the presence of overlooked pathogens in the field.

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