Scientific Article

Association of *Ureaplasma diversum* with reproductive disease in cattle.

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Abstract

AIMS: To examine the association between the detection of *Ureaplasma diversum* in vaginal swabs from dairy cows in north western Spain with the diagnosis of granular vulvovaginitis (GVV) and reproductive performance, and the association with subclinical endometritis in slaughterhouse material. In addition the presence of this microorganism in cases of abortion was investigated

METHODS: First, the participation in this study was offered to farms in the region. 106 accepted and, among those, 40 herds were randomly selected. Ten animals per farm were randomly sampled and their vaginal swabs were mixed in one pool per farm to detect the presence of *U. diversum* by qPCR. Five out of 40 herds were randomly selected and the vaginal swabs of the 10 animals initially sampled were individually tested by qPCR for *U. diversum* (n = 50). The presence of GVV lesions and the reproductive efficiency of the animals (based on the number of necessary inseminations to achieve a gestation in two subsequent pregnancies) were registered and analysed in relation to the presence of *U. diversum*. Secondly, 100 uteri of cattle obtained in the slaughterhouse were tested to study the association between *U. diversum* and subclinical endometritis (SE). Finally, diagnosis of abortion was offered to farms in the region. Sixteen farms with abortion problems submitted samples and we studied the presence of this bacterium evaluating the best tissue for diagnosis.

RESULTS: A high herd prevalence (39/40) of *U. diversum* was found and it was also the most prevalent pathogen in the samples from slaughterhouse (5/100). Statistical associations were found between the presence of DNA with granular vulvovaginitis and subclinical endometritis. Reproductive efficiency was poorer in *U. diversum* positive animals. *U. diversum* was also diagnosed in 4/16 farms with abortion problems and farms presented moderate abortion rates, but abortions occurred over a long period of time, and liver appeared to be the best tissue for detecting *U. diversum* DNA in the foetuses analysed.

CONCLUSIONS AND CLINICAL RELEVANCE: The infection was present in most of herds and it was statistically associated with GVV, SE and poor reproductive performance. It was also detected in abortions and the liver may also be an additional tissue to be considered in the diagnosis of *U. diversum* abortion by PCR. The possible association with different diseases in the same area suggests that different presentations should be considered when studying the implications of *U. diversum* on the reproductive diseases of cattle.

Keywords: *Ureaplasma diversum*; abortion; vulvovaginitis; endometritis; infertility; cattle

AI	Artificial insemination
GVV	Granular vulvovaginitis
qPCR	Quantitative real-time polymerase chain reaction
SE	Subclinical endometritis

Introduction

Ureaplasma diversum has been related to different reproductive diseases in cattle with a variable degree of evidence and this bacterium is found both in sick and asymptomatic animals (Mulira *et al.* 1992; Sanderson *et al.* 2000; Petit *et al.* 2008; Argue *et al.* 2013). Initially, this bacterium was associated with Granular Vulvovaginitis (GVV), a disease characterized by hyperaemia and granularity in the dorsolateral vulva. Statistically significant associations between this bacterium and GVV have been reported (Doig *et al.* 1979; Mulira *et al.* 1992; Gaeti *et al.* 2014; Azevedo *et al.* 2016), and these lesions have been experimentally reproduced after inoculation (Doig *et al.* 1980). The disease presents acute and chronic forms differentiated by their severity and the presence of purulent discharge (Doig *et al.* 1979), but animals do not usually show other signs of illness (Miller *et al.* 1994).

U. diversum has also been related to infertility, endometritis and salpingitis (Kreplin *et al.* 1987; Smits *et al.* 1994). Nevertheless, the involvement of this bacterium in cattle fertility remains unclear due to the lack of evidence for a causal mechanism, and some studies have not detected any problems in some infected farms (Reid *et al.* 1989; Petit *et al.* 2008). It remains plausible that infection is not truly pathogenic or that other factors are required for pathology to be induced (Sanderson *et al*. 2000).

U. diversum can also cause abortion in the mid to final trimester (Miller *et al.* 1994; Anderson 2007). However, this pathogen is not widely considered in the differential diagnosis of cattle abortion, and these abortions are generally considered to be sporadic. However, it is not entirely clear whether the lack of detection is sometimes due to the fact that it is a rare abortifacient or because it is not an aetiology frequently considered in the laboratory diagnosis. Anyway, a high proportion of abortions has been attributed to *U. diversum* in some

regions (Maxie 1986; Syrjälä *et al.* 2007), while not in others (Kirkbride 1993). This may be due to true differences in the incidence of *U. diversum* between regions, or due to differences in the sensitivity of detection of this pathogen between the testing strategies employed. (Miller *et al.*1994). Thus, determining its epidemiological performance and more convenient procedures to successfully achieve a diagnosis would be useful to understand the relevance of this bacterium as an abortifacient agent.

Published research generally gives low relevance to this infection and it is not a commonly considered microorganism in the veterinary practice. Most of the studies have focused separately on the potential association between *U. diversum* and some of the aforementioned diseases. However, the full effect of the bacterium in a herd may be underestimated if the full range of syndromes is not considered as a whole and it is also possible that *U. diversum* has different presentations depending on the herd or region. Consequently, it would be valuable to study the association of this bacterium to different diseases in a same population. In order to get an overall view of this situation, we described the presence of DNA of *U. diversum* at three levels: 1) cattle farms, 2) at slaughterhouse and 3) in abortion cases. Our aim was to examine the association between the detection of *U. diversum* in vaginal swabs from dairy cows in north western Spain with the diagnosis of granular vulvovaginitis and reproductive performance, and the association of this infection with subclinical endometritis (SE) in slaughterhouse material. In addition the presence of this microorganism in cases of abortion was investigated.

Materials and methods

Study area.

This study was performed in the province of Lugo (NW Spain), which accounts for approximately 18% of production and 20% of dairy cows in the country. The region accounts for about 260,000 cows and 14,000 herds, but most of them have a reduced number of animals and the average herd-size is 18 cows per farm. Herds with more than 20 animals constitute approximately 40% of the farms and include 72% of the census. Husbandry is intensive and the Holstein-Friesian is the most common dairy breed.

Study design and samples collected

U. diversum in cattle farms

The participation in the study was offered to dairy farms in the study area by contacting with servicing veterinarians. A total of 106 farms agreed to participate and among them, 40 (38%) were randomly sampled in April 2015. Farm sizes ranged 24-116 animals and breeding was performed by artificial insemination (AI). In each farm, fertility was assessed as the proportion of eligible cows that are confirmed pregnant to insemination within 21 sequential days. The annual mean of this value was defined as the fertility rate of the herd. The development of this study can be differentiated in two parts. First, the selected farms were sampled for detecting the presence of *U. diversum* infection. Thus, a fixed sample size of 10 animals older than 26 months was taken in each farm, regardless of their pregnancy status. This allows for the detection of disease if prevalence is over 25% and population size lower than 120 animals (Thrusfield 2007).

In each animal, a vaginal swab was collected and eluted into 1mL of PBS to detect *U. diversum* DNA. In this part, the vaginal swab elutions from the same farm were combined to obtain a pooled sample per farm (n = 40). These pools were analysed by quantitative real-time PCR (qPCR) to label the farm as positive or negative to infection with *U. diversum*.

The presence of lesions compatible with GVV (Doig *et al.* 1979; Mulira and Saunders 1994) was recorded in each sampled animal. These lesions were defined as the presence of white spherical nodules in the dorso-lateral wall or in the dorsal corner of the vulvar mucosa which may be concurrent with hyperaemia, thickening or purulent discharge (Doig *et al.* 1979).

In the second part, five out of the 40 initially sampled farms were randomly selected. In this case, the 10 elutions of vaginal swabs that were available per farm were individually analysed in order to study the individual presence of *U. diversum*, by qPCR (n = 50 animals). The association between the detection of *U. diversum* DNA and the presence of GVV lesions was studied. Given that BoHV-1 may produce similar lesions to GVV, serum samples from these 50 animals were also collected to detect antibodies against BoHV-1 using a commercial blocking ELISA kit (IBR gB X3; Idexx Laboratories, Inc.; Westbrook; ME; USA).

Finally, the reproductive indicators of these 50 animals were collected from the farm records. At the time of sampling (April 2015), the reproductive status (pregnant, non-pregnant

or inseminated with no confirmation of gestation), number of inseminations required to achieve pregnancy and the incidence of any reproductive associated events during this period (abortion, metritis, etc.) was recorded. One year later (April 2016), the same information was collected. Thereby, it was possible to monitor the overall reproductive performance of the animals during this period including the moment of sampling. The success of AI was evaluated after 30-40 days by echography.

U. diversum in uteri collected from slaughterhouse

A total of 100 uteri of dairy cattle were randomly sampled between January and March 2014 in a slaughterhouse in the study area (Lugo, Spain). Individual data, reproductive performance of the cows or reasons for culling were unknown and animals were sampled independently from these characteristics. Two swabs were taken from each uterus. One swab was destined for microbiological analysis by aerobic culture, and the other one was used to detect the presence of *U. diversum* DNA by qPCR. Samples of uterine content were also collected for cytological analysis by introducing a cytobrush into the caudal tract of the uterus horns and rolling over the endometrial surface to determine the presence of SE. Thus, the purpose of this part was to evaluate the prevalence of detection in the slaughterhouse and study the association with SE,

U. diversum in abortion cases

The presence of *U. diversum* was studied in cases of abortion. For this purpose, submissions for laboratory diagnosis were investigated. The participation in the study was therefore voluntary and the diagnosis of bovine abortion was offered to farms in this region that applied for it from 2013 to 2015. The number of cases summed a total of 16 farms and they accounted for 25 bovine foetuses. Cattle belonged to dairy farms (Holstein-Friesian). Farms were participants of control programs for BVD, IBR and *Neospora caninum.* The participation in such programs and the status of the farms were verified by contacting with the veterinarians in charge.

Samples consisted of foetuses, placentas and vaginal swabs of the aborted cows. They were collected by veterinarians who worked in these farms and submitted for laboratory diagnosis. At the laboratory, foetuses were submitted to standard examination and necropsies were performed to obtain foetal organs. As a result, the specimens available for diagnosis were foetal organs, including liver, lung, kidney, brain, spleen and abomasal contents, in addition to the maternal placenta and vaginal swabs.

The detection of *U. diversum* was performed in two steps. First, farms were screened by qPCR using three types of samples: pooled vaginal swabs (n = 16), pooled foetal organs (n = 25), and abomasal contents (n = 25). When the DNA of *U. diversum* was detected in some of these samples, the farm was considered positive. Then, all available specimens from this case were then analysed separately by qPCR. The foetus was considered positive when DNA of *U. diversum* was found in foetal tissues. The presence of other potential abortifacient agents was analysed by using microbiological culture and qPCR. Culture allowed the detection of aerobic and microaerophilic bacteria with no special requirements to grow. qPCR was performed using commercial validated kits and they were used to detect: BoHV-1, *Campylobacter* spp., *Coxiella burnetii, Leptospira* spp., *N. caninum,* and BVDV-1/BVDV-2 and *Salmonella enterica* spp. This diagnosis scheme was based on the available bibliography on the topic using target tissues recommended for each pathogen (Anderson 2007). The diagnosis scheme allows the detection of a wide variety of abortifacient pathogens. Venereal pathogens like *Trichomonas foetus* were not taken into consideration since farms perform AI. *Brucella* spp. was not considered since the study area is officially declared free of bovine brucellosis.

During the submission of samples, farm veterinarians were asked to fill an epidemiological survey and report the following reproductive data: duration of problems (expressed as the period of time since the farmer noticed problems in his farm and using a four category variable: < 1 month, 1-4 months, 5-12 months; > 12 months), annual percentage of abortions in the farm (number of animals aborted divided by the total pregnant animals in the last year); calving interval (number of days between calvings of the animals in the farm) and percentage of cows that received > 2 AI. Breeding was performed by AI.

Laboratory analysis

Extraction of DNA

The extraction of DNA was performed using a commercial extraction kit (NucleoSpin Tissue; Macherey-Nagel; Düren; Germany). In the different parts of the study, 4 types of materials were extracted: individual vaginal swabs, pooled vaginal swabs, foetal tissues, pooled foetal organs.

The pooled vaginal swab samples were made by mixing 200 µL of elution from each aborted animal submitted per farm. Both in individual and pooled vaginal swabs, the quantity of sample used as starting material was 200 µL.

The pooled foetal organ samples were made by mixing the liver, lungs, kidneys and spleen of each foetus. 0.1 g of each organ was randomly selected and suspended in 16 mL of phosphate buffer saline. The mixture was homogenized using a mechanical homogenizer (Seward; Worthing; UK). 200 µL was also the starting quantity of material for the extraction. When the tissues (placenta, spleen, liver, kidneys, brain and lungs) were individually analysed, extraction was performed using 25 mg of the tissue.

The subsequent steps of the extraction procedure were performed following the manufacturer's instructions for tissue samples and for all types of samples.

qPCR

qPCR was used to detect presence/absence of *U. diversum* DNA in the pooled vaginal swabs, in the swabs from uteri collected from the slaughterhouse and in the samples in the screening step of the study in abortions.

In cases of abortion that tested positive for *U. diversum*, qPCR was used for the individual vaginal swabs and all available foetal samples. A commercial qPCR kit (UreDiv dtec-qPCR Test; GPS; Elche; Spain) was used following the manufacturer's instructions for *U. diversum.* A sample was considered positive when a signal exceeding the threshold was detected before cycle number 40. Other abortive pathogens were tested using commercial PCR kits. The thermocycler used was an ABIPRISM 7500 (Thermo Fisher Scientific, Waltham, USA).

Microbiology and cytology

Swabs were inoculated onto blood agar plates and incubated at 37º C for 72 h under aerobic conditions performing daily readings. In addition, two blood-agar plates were inoculated with a sample of 30 µL of foetal abomasal contents per plate. One plate was incubated at 37º C in aerobic conditions and the other in microaerophilic conditions using microaerophilic atmosphere generator sachets (GenBag; Biomerieaux; Marcy-l'Étoile, France) (Campero *et al.* 2003), performing daily readings for 72 h. This allows the detection of pathogenic bacteria with no special requirements for growth, both aerobic and microaerophilic (e.g. *Campylobacter* spp.). The most important reproductive bacterial abortifacients that may not grow in these conditions were screened by qPCR. Bacteria were identified by using Gram staining and biochemical tests.

Smears of samples obtained using the cytobrush were stained with Diff-Quick (Química Aplicada; Tarragona; Spain) and observed at 400x magnification. A total of 250 cells were counted for each smear, and the percentage of polymorphonuclear cells (%PMN) was calculated. Subclinical endometritis was considered when %PMN was over 5% (Gilbert *et al.* 2005).

Statistical analysis

In cattle farms, the association between GVV lesions and the presence of *U. diversum* DNA and seropositivity to BoHV-1 was studied by using the chi-square test.

The reproductive efficiency of the 50 animals followed-up was calculated using their data from their last two pregnancies before April 2016. Animals that achieved a gestation with three or fewer AI in the previous two pregnancies in the absence of any other reproductive events (abortion, metritis, etc.) were considered with good efficiency; otherwise they were considered with poor efficiency. Animals were excluded from the analysis due to three causes. 1) Animals with abortion or metritis, because these conditions may affect the efficiency to achieve a gestation regardless of the infection with *U. diversum*. 2) Animals eliminated from the farm between April 2015 and April 2016. 3) Animals whose last pregnancy with less than 3 AI could not be proved by April 2016 had to be excluded from the analysis since it was not possible to assign them to a group. As a result, 31 animals were finally included in the analysis. The purpose of the classification was to study the differences in the percentage of animals infected with *U. diversum* between the groups with good and poor reproductive efficiency. Thus, a mixed logistic regression was performed with reproductive efficiency as the dependent variable and using the positivity to *U. diversum* and the age of animals (stratified in 3 groups: 26-35 months; 36-48 months and > 48 months) as exploratory variables. The herd was included as a random effect. In order to explore a potential masking of the effect of *U. diversum* due to the exclusion of animals, an additional mixed logistic regression was conducted with missing animal as the dependent variable, positivity to *U. diversum* as fixed factor and herd as random factor.

In the samples from the slaughterhouse, the association between the presence of SE and the presence of *U. diversum* DNA was analysed by a simple logistic regression.

Statistical analyses were performed with R software (R Core Team 2014). chisq.test, glm() and glmer() from the "lme4" package (Bates et al., 2015). The function confint() was used to obtain the confidence intervals.

Results

U. diversum in cattle farms

Thirty-nine out of the 40 pooled vaginal swabs samples resulted positive for *U. diversum* (97.5%). In the five farms selected for the analysis of individual vaginal swabs, a high range of annual rolling fertility rates (22 to 49%) was observed (Table 1). In these farms, the animals with GVV-like lesions ranged from 2/10 (20%) to 9/10 (90%) and the animals positive for *U. diversum* ranged from 3/10 to 7/10. Twenty-five out of 50 (50%) of the individually tested animals presented lesions compatible with GVV, and 25/50 (50%) resulted positive for *U. diversum* by qPCR (16/25 animals with GVV-like lesions (64%) and 9/25 animals without GVV-like lesions). A significantly higher percentage of lesions was found in animals positive for *U. diversum* ($p = 0.047$; OR: 3.1; CI95%: 1.9-11.8). In contrast, only 3/50 (6%) animals were seropositive to BoHV-1 with no association with GVV-like lesions ($p = 0.234$), since they were absent in the three animals.

Among the 50 animals which were individually analysed, 2/50 were removed from the farm (one positive to *U. diversum*), 3/50 presented other reproductive problems (2 positives) and 14 were removed because their last pregnancy could not be verified or their records were incomplete (8 positives). As a result 31/50 animals were studied. A significantly higher percentage of animals with poor reproductive efficiency was found in animals which were positive to *U. diversum* (57%; Table 2). No statistical differences were found between exclusion from the study and *U. diversum* status ($p = 0.384$)

U. diversum in uteri collected from the slaughterhouse

At least one microorganism was detected in 18/100 (18%) uteri. The predominant microorganisms were *U. diversum* (5/100), *Escherichia coli* (4/100), *Aerococcus urinae* (3/100), *Trueperella pyogenes* (2/100) and *Moraxella* spp. (2/100). In five out of 100 samples, more than one microorganism was detected. *U. diversum* was solely detected in 3/5 (40%) cases, but other bacterial species were isolated in the two remaining cases (*E. coli*, *Streptococcus* uberis and *Enterococcus durans*).

Nineteen (19%) samples presented SE. At least one microorganism was detected in 13 out of 19 samples with SE (68%) and 6/18 (33%) samples with microorganisms did not present SE. *U. diversum* was detected in 5 cases (5%): 3/19 (16%) animals with SE and 2/81(2%) animals without SE. In 2/3 cases in which *U. diversum* and SE were detected, *U.*

diversum was the only bacterium detected. A statistical association was found between SE and presence of *U. diversum* (p = 0.036; OR: 7.4; CI95%: 1.14-47.96)

U. diversum **in abortion cases**

Four out of 16 farms (25%) with abortion were positive to *U. diversum* since the bacterium was detected by qPCR in 4/16 pool vaginal swabs (25%). *U. diversum* was the only microorganism detected in 3/4 farms, and *Campylobacter* spp. was also detected in the remaining farm. The percentage of cows with more than two AI ranged between 21-39% and the duration of problems according to farmers was longer than 5 months in all cases (Table 3).

The positive farms submitted a total of seven foetuses. *U. diversum* was found in the pooled organs of 6/7 (86%) foetuses. No other pathogens were detected in these six foetuses. The remaining foetus tested positive for *Campylobacter* spp. but negative to *U. diversum.* The individual organs could not be analysed in one of the six positive foetuses. In the other five, *U. diversum* was detected in the liver of all the foetuses. Lung samples were positive in two foetuses. The placenta could only be obtained in four out of six cases, but 3/4 were positive for *U. diversum*,

Discussion

Infection with *U. diversum* has been tradicionally considered to be a sporadic problem in cattle with limited relevance (Miller *et al.* 1994; Petit *et al.* 2008). However, the relationship of the infection with diseases has been mostly studied separately. Thus, the effect of *U. diversum* may be better estimated were consideration given to all the potential effects of this bacterium included in analyses. Our results are consistent with this hypothesis since we found a widespread presence of the bacteria statistically associated with different reproductive diseases in the herds analysed. Nevertheless, it should be noted that farm inclusion depends on the willingness of farmers to join and this might have a role in the representativity of the whole region.

Firstly, the infection was statistically associated to GVV, in accordance with other studies (Doig *et al.* 1980; Gaeti *et al.* 2014). We also discard that the lesions we found were related to BoHV-1 because antibodies against BoHV-1 were seldom detected. Thereby, the presence of lesions may be used as a rapid first screening to suspect of an *U. diversum* infection in a farm However, a subsequent confirmatory analysis should be conducted to verify the presence of this bacterium and discard the presence of BoHV-1 when its status is

unknown. Nevertheless, at animal level, this approach would be useless since positive animals without lesions were also detected. These healthy but infected animals might be the consequence of the healing of the vulvar mucosa, but they still preserve an important bacterial population and they may serve as asymptomatic carriers. Different studies have attempted to analyse if some factors like body condition or serum blood nitrogen could be related to the expression of the disease, but clear associations could not be determined (Rae *et al.* 1993; Sanderson *et al.* 2000).

A relationship between farm fertility rate and *U. diversum* could not be determined since the vast majority of farms were positive. Nevertheless, the percentage of animals with poor reproductive efficiency was found to be significantly higher in animals that tested positive to *U. diversum*, which required a greater number of AI to become pregnant. A causal association cannot be inferred, but this finding might suggest a potential implication of the pathogen in cattle fertility, and it would be consistent with other reports, where the bacterium was found to be associated with worse reproductive performance in one way or another (Doig *et al.* 1979; Kreplin *et al.* 1987). However, there are some limitations in our study that must be noted. The precise moment of infection was unknown so we considered the reproductive efficiency as a variable related to the long-term effects on fertility defining two subpopulations of animals. However, factors not controlled in the study that may appear after sampling and affect reproductive performance might bias the results. This approach may also produce some bias in terms of inclusion of animals and mask part of the infection impact. In particular, acute or chronic infections may have different effects in fertility and the consequences of the infection may also vary depending on the reproductive status of the animal when the infection occurs. Moreover, animals removed from the study may constitute a source of bias, although we did not find a significant percentage of *U. diversum*-positives among the removed animals. In any case, the effects that *U. diversum* may potentially have in the fertility of the studied farms would not be dramatic, as we have seen that some infected herds presented good fertility rates despite being positive. Indeed, other authors have indicated that *U. diversum* is likely to have a minor impact on cattle infertility (Petit *et al.* 2008), and infection has also been reported in herds with normal conception rates (Reid *et al.* 1989).In light of these discrepancies, the effects may be circumscribed to determined conditions or animal statuses. Prospective studies considering differences in fertility between herds or groups of infected/non-infected animals may aid to rightfully infer potential associations.

Our results show that this microorganism may be present in uteri with a statistical association to SE. Some authors have suggested that the presence of this bacterium in the vulva may be normal, but its detection in the cervix or uterus may indicate a pathologic condition (Ball and McCaughey 1979). Experimental inoculations in uteri have been reported to lead to failure in the establishment of pregnancy in cattle (Gale 1987; Kreplin *et al.* 1987). The microorganism may alter the normal patterns of prostaglandins in the uterus, which may affect implantation and the maintenance of gestation (Kim *et al.* 1994). However, further studies are necessary to unravel its implication in this process as well as the potential sources

We found a considerable percentage of farms with abortion problems in which this microorganism was detected in foetuses (25%). This percentage does not intend to be representative of the whole population of study due to the sample size and the sampling design. In all the *U. diversum*-positive foetuses, no additional pathogens were detected. Some authors reported that it is relatively uncommon to only identify *U. diversum* in an abortion (Murray 2012). However, our results show that, the sole detection of this microorganism is also a possible scenario. In order to discard the presence of other pathogens we have used techniques that have been reported to be useful in the diagnosis of bovine abortion in literature. Nevertheless, problems of sensitivity could still exist if pathogens were not present at the time of examination. Posterior feedback with veterinarians did not prove any evidence of this situation and we consider it unlikely.

The need for a meticulous culture of this microorganism may affect the sensitivity of diagnosis based on culture techniques (Nicholas *et al.* 2008). Therefore, PCR represents an important tool because it does not require viability and it has been reported to be highly sensitive and specific in bovine vaginal samples (Marques *et al.* 2013). Abortion due to *U. diversum* has been related to foetal ingestion of contaminated amniotic fluid, resulting in chorioamnionitis, bronchopneumonia and conjunctival hyperplasia (Miller *et al.* 1983; Ruhnke *et al.* 1984). Consequently, the diagnosis of abortion has generally focused on the isolation of the microorganism in quasi-pure culture of foetal lung or abomasal contents (Ruhnke *et al.* 1984; Anderson 2007). However, when we analysed different tissues by qPCR, the foetal abomasal content tested negative in all the positive cases and if only lung was tested, and not liver, the presence of the bacteria would not have been detected in three cases. Consequently, although the number of tested foetuses in this study was low, it seems reasonable to consider the liver as an additional tissue to be evaluated in the diagnosis of *U.*

diversum-related abortions, in order to avoid the possible underdiagnosis of this microorganism.

Due to the limitations of our study, it cannot be concluded that *U. diversum* was the cause of the abortions. Moreover, it should be noted that the analysed farms implemented control programs for other reproductive pathogens (BVD, IBR and *N. caninum*). Therefore, our proportions may not be representative of the actual rate of abortions caused by *U. diversum* in a population, but they indicate that the risk of these abortions may occur exists and they might gain relative importance when other pathogens are controlled. Positive farms presented a long duration of the episodes of abortion and an important percentage of cows required more than two AI to get pregnant and, although it cannot be proved that *U. diversum* was the cause of the abortion or the poor fertility observed, a possible chronic effect in farms can be an epidemiological scenario to be considered. This presentation has not been comprehensively investigated yet, so it would be valuable that further studies consider the potential implication of this pathogen as a cause of mild, but persistent problems in farms which might be accompanied by other manifestations in the reproductive performance of the herd.

The main aim of this article was to show that *U. diversum* may be statistically related to different reproductive problems in the same population. Causal mechanisms cannot be inferred but the associations found in this study indicate that there is still a need for an adequate characterization of the involvement of *U. diversum* in some of these diseases. In our opinion that in farms with persistent reproductive problems or the occasional occurrence of abortions, the consideration of *U. diversum* in the differential diagnosis is justifiable, at least after excluding other pathogens of importance. We recommend the observation of GVV lesions as a possible rapid indicator to suspect of *U. diversum* infection in a herd with appropriate considerations and the liver was found to be a good tissue for the diagnosis of abortion by PCR, which may be used in addition to others typically used.

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Table 1. Descriptive results of the animals (n = 50) included in the follow-up by farm

Farm	GVV-lesions (%)	Positive to *Ureaplasma diversum*		Seropositive to BoHV-1		Fertility rate	Good-poor reproductive efficiency (n = 31)[a]
		Total (%)	With GVV-lesions/ total positive (%)	Total (%)	With GVV-lesions/ total positive (%)		
A	2 (20)	4 (40)	1/4 (25)	2 (20)	0/2	26%	5-0
B	6 (60)	6 (60)	5/6 (83)	0	0	22%	6-1
C	3 (30)	5 (50)	3/5 (60)	0	0	39%	4-1
D	9 (90)	3 (30)	3/3 (100)	0	0	49%	3-4
E	5 (50)	7 (70)	4/7 (57)	1 (10)	0/1	36%	2-5

[a] Reproductive efficiency only could be evaluated in 31 animals

Table 2. Results of the mixed logistic regression of the reproductive efficiency by presence of *Ureaplasma diversum* and age (n = 31)

Dependent variable	Factors	Category	Poor efficiency/total in category (%)	p	OR[a] (CI95%[b])
Reproductive efficiency	Presence of *U. diversum*	Negative[c]	3/17 (18)	[c]	[c]
		Positive	8/14 (57)	0.029	6.36 (1.40-28.88)
	Age	26-35 months[c]	3/9(27)	[c]	[c]
		36-48 months	1/5 (20)	0.535	0.41 (0.01-12.32
		> 48 months	4/17 (24)	0.981	0.97 (0.12-4.09))

[a] : Odds ratio; [b] Confidence interval at 95%; [c] : reference category

Table 3. Characteristics of farms with abortions with detection of *Ureaplasma. diversum*.

Farm	Annual abortion rate	Duration of problems[a]	Calving interval (days)	% cows > 2 AI	Detection of other pathogens in farm	Positive foetuses to *U. diversum*/ total)
1	15%	5-12 months	438	32	No	2/2
2	10%	5-12 months	441	21	No	2/2
3	12%	1 year	439	39	*Campylobacter* spp.	1
4	NA	1 year	NA	NA	No	1/1
Negative farms[b] (range)	20% (12-36)	1-4 months (50% of farms)	430 (411-456)	20 (18-43)	[c]	
Median in the region[d] (range)	5% (1-32%)	NA	415 (356-511)	NA (15-48)		

[a] : time perceived since farmers believe there are abortion problems in the herd (<1 month; 1-4 months; 5-12 months; > 1 year)

[b]: farms that submitted abortions but were negative to *U. diversum* (n = 12). Data is expressed as average or more frequent result (in case of duration)

[c] :Pathogens detected in *U. diversum*-negative farms: BVDV-1, *Neospora caninum, Campylobacter* spp., *Trueperella pyogenes* and *Streptococcus* spp.

[d]: Source for the data: Diaz-Cao, 2016

NA: not available