Ruminant pestiviruses in North Africa

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ABSTRACT

Ruminant pestiviruses are widely distributed worldwide, causing congenital disease and massive economic losses. Although ruminant production is an important economic sector in North Africa, the knowledge about pestiviruses is scarce. The present study aimed at assessing the presence of *Pestivirus* in cattle in Algeria, and to review the data available on ruminant pestiviruses in North Africa. A cross-sectional study was conducted on dairy farms from North-Western Algeria. Blood samples from 234 dairy cattle from 31 herds were collected. All sera were analysed for the presence of antibodies using a commercial iELISA. The presence of *Pestivirus* RNA was also assessed by using a Reverse Transcription-PCR, and PCR-positive samples were sequenced. Risk factors related to *Pestivirus* infection were also analyzed. The review of the presence of ruminant pestiviruses in North Africa was performed using a systematic search and compilation methodology of the peer-reviewed literature available in order to identify gaps of knowledge for future research. The seroprevalence at population and farm levels obtained in the present study (59.9% and 93.5%, respectively) concur with data reported in neighboring countries. Risk factors associated with *Pestivirus* infection in cattle were the presence of sheep in the herd and the animal category (cow **vs heifer**). Furthermore, we confirmed the presence of BVDV-1a in Algeria. The scarce data suggest an endemic epidemiological scenario of *pestivirus* in livestock. The lack of studies about the epidemiology and molecular variability of ruminant pestiviruses in livestock and wildlife in North Africa is of concern for animal health and wildlife conservation, and needs to be addressed.

Key words: Algeria; BDV; BVDV; livestock; North Africa; *Pestivirus*

1. Introduction

The genus *Pestivirus* comprises four traditionally recognized species of enveloped, positive-sense single-stranded RNA (12.3 kb) viruses: Bovine Viral Diarrhoea Virus (BVDV) -1, BVDV-2, Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV) (Schweizer and Peterhans, 2014). Recently, the International Committee on Taxonomy of Viruses (ICTV) has recognized eleven species within this Genus (*Pestivirus* A to K) (ICTV 2018). In adult ruminants, horizontal *Pestivirus* infection usually causes a mild disease and short viremia (7-15 days) that ends with the outcome of humoral response. Vertical transmission of the virus will cause fetal resorption, abortion, stillbirths or malformations of the fetus. However, if the infection by *Pestivirus* A (BVDV-1) or *Pestivirus* B (BVDV-2) occurs between the second and fourth month of gestation in cattle, or before the second month in sheep in the case of *Pestivirus* D (BDV), the infection may lead to the birth of Persistently Infected (PI) animals that are immunotolerant to the virus (Schweizer and Peterhans, 2014). These PI individuals are the main source of spread and persistence of ruminant *Pestivirus* in livestock. Ruminant pestiviruses are distributed worldwide causing congenital disease and entailing massive economic losses (OIE, 2019).

Algeria faces a huge deficit in dairy and meat production, triggering a significant annual import expenditure, which amounted to US$ 2.045 billion for milk and US$ 0.307 billion for meat in 2014 (Kardjadj and Luka, 2016). A massive surge in food demand has occurred in Maghreb countries, particularly in animal products (meat and milk), implying the need for a "Livestock Revolution" (Sraïri et al., 2013). Red meat marketed and consumed in Algeria consists essentially of mutton and beef. According to the official cattle census of Algeria (MADR, 2017a) there was a total of 1.89 million bovine heads in 2017. The bovine production is one of the main sources of both meat and milk in Algeria, playing a vital role in food security. The 33% of the national consumption is provided by national production. Cattle farming is

generally structured in small to medium-sized workshops dedicated mainly to milk production (Mouffouk, 2014). The cattle population is composed of imported cattle (mainly Holstein and Montbéliarde from Europe), local cattle (the local breed "*Brune de l'Atlas*") and crossbreed cattle (crosses between the local and the introduced breeds). The population of small ruminants in Algeria comprises more than 31.2 million heads (26.4 million sheep and 4.8 million goats) (MADR, 2017b). Sheep meat production amounts to 325,000 tons (Business France, 2018). Animal health improvement is necessary in Algeria, especially regarding reproductive disorders (abortive pathogens). *Pestivirus* seroprevalence in ruminants has been rarely assessed in the country and to our knowledge there is only one study in cattle, one in sheep and one in dromedary camels (Derdour et al., 2017; Feknous et al., 2018; Saidi et al., 2018). The only study in cattle reported a seroprevalence of 1.39% in the North-centre of the country (Derdour et al., 2018). In fact, cattle are not routinely vaccinated against BVDV in Algeria and the knowledge about epidemiology and molecular epidemiology of *Pestivirus*, especially *Pestivirus* A-B, in the whole North-African region is scarce. The present study aimed to study the genetic diversity of *Pestivirus* circulating in cattle herds in Algeria, to determine the risk factors for *Pestivirus* A-B infection in Algeria, and to review the available data of ruminant pestiviruses in North Africa.

2. Materials and Methods

2.1. Animals and samples

A two-stage sampling survey was carried out in North-Western Algeria (Tiaret province; 35°22'10.1"N 1°19'47.7"E) between June 2018 and August 2019. The Tiaret province is located approximately 160 Km from the Mediterranean coasts and covers an area of 20,399 km2 including part of the Tell Atlas in the North and the highlands in the centre and South.

Temperature ranges from 6 to 25ºC and precipitations from 4 to 69 mm through the year. As in the whole country, smallholder dairy systems are the dominant organization in the region. The Tiaret Veterinary Office provided a list of all cattle herds registered in the province, which included information of the herd owner, the address or number of animals. The sampling frame included 289 dairy cattle herds.

For the first stage of sampling (sampling of herds), the sample size for disease detection was calculated based on the following formula (Dohoo et al., 2003):

$$n_1 = \left(1 - (1 - \alpha_1)^{\frac{1}{D_1}}\right) \times \left(N_1 - \frac{D_1 - 1}{2}\right)$$

where α_1 was the confidence level (set at 95%), D_1 was the minimum number of infected herds (estimated as $D_1 = Prev_1 \times N_1$), where $Prev_1$ was the minimum herd prevalence to be detected (set at 10%), and N_1 was the population of herds (which in our case were 289 dairy herds). The estimate of n_1 was 29 herds. No formal random process was used for the selection of herds. Instead, from the list, a herd was randomly selected, and the herd owner was contacted, and asked, first a) whether they complied with the inclusion criteria, and then b) whether they were willing to participate. The process was repeated until the number of herds needed for the first stage was completed. The inclusion criteria comprised that the herd had at least one female animal above six months, and that the milk was not only for own consumption (i.e. some of the milk was sold). This age category was selected to avoid interference as much as possible the detection of maternal antibodies in the seroprevalence studies (Chase et al., 2008).

For the second stage (sampling of animals within herds), the sample sizes for disease detection were also calculated based on the formula by Dohoo et al (2003):

$$n_{2i} = \left(1 - (1 - \alpha_2)^{\frac{1}{D_{2i}}}\right) \times \left(N_{2i} - \frac{D_{2i} - 1}{2}\right)$$

where α_2 was the confidence level (set at 95%), D_{2i} was the minimum number of infected animals in herd i (estimated as $D_{2i} = Prev_2 \times N_i$), where $Prev_2$ was the minimum within-herd

prevalence to be detected (set at 30%), and N_{2i} was the population size of herd i (size of herds selected in stage 1 varied between 7 and 62). The estimate of n_2 varied between 4 and 8. The sampling of animals within herds (second stage) was also random, although because of the lack of proper sampling frames, no formal random process was used either. Random animals in the herd were selected until the number of animals needed for the second stage was completed. However, because of logistics problems, the number of samples per herd could not always be completed, and therefore some extra **convenience** samples were collected in some of the remaining herds, and also a few extra herds were sampled. Sample sizes for the two stages were calculated using Epitools (Sergeant, 2018).

The sampled population was composed of 234 dairy cattle aged between 9 and 180 months, in semi-intensive husbandry system. Holstein, Montbéliard and crossbreed were the most common breed and the size of the sampled herds varied from ten to a hundred individuals. Blood samples (5 ml) were collected by qualified private and state veterinarians, from the coccygian vein on sterile dry vacutainer tubes, using disposable needles, and was immediately sent on ice to the local laboratory. The sera were extracted by centrifuging the samples at 1200g for 10 min and were then stored at −20°C, until tested. All samples were then sent on dry ice to the Animal Health Research Centre (CReSA, IRTA-UAB, Bellaterra, Spain) where the serological and molecular analyses were performed. The study was approved by the ethical review board of the Veterinary Institute of the University Ibn Khaldoun (Tiaret, Algeria).

2.2. Serological and virological analyses

The presence of antibodies against *Pestivirus* p80 protein was determined using a commercial iELISA (IDEXX, Montpellier, France) in accordance with manufacturer's instructions. All sera were also analysed for the presence of *Pestivirus* RNA using a Reverse

transcription-PCR (RT-PCR). Total viral RNA was extracted directly from 200µl of sera using the commercial kit IndiMag® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) according to the manufacturer's instructions. The RT-PCR was performed using primers 324 and 326 (Vilček et al., 1994) and a commercial kit (One-Step PCR kit, Qiagen Inc., Hilden Germany). *Pestivirus* positive amplicons were sequenced, and the 5' untranslated region (5'-UTR) was characterized.

2.3. Prevalence and risk factors

In order to account for effect of the two-stage sampling design in the calculation of prevalence and confidence intervals, the R package survey (Lumley, 2020) was used.

Also, to evaluate the effects of the sensitivity and specificity of the commercial iELISA on the prevalence estimation, the true prevalence (TP) of disease (and corresponding 95% CI) were calculated according to the following formula (Dohoo et al., 2003):

$$TP = \frac{AP + Sp - 1}{Se + Sp - 1}$$

where AP was the apparent prevalence, Sp was the specificity of the test (0.97; Hanon et al., 2017), and Se was the sensitivity of the test (0.60).

Risk factors related to *Pestivirus* infection were evaluated by studying several individual and herd traits, such as breed category (pure for Montbéliarde, Holstein, Brown of the Alps and Fleckvieh; and crossbreed for crosses between local breed and imported dairy cow, commonly, Holstein and Monbéliarde), animal category (heifers and cows), herd size, mixed herd (cattle mixed with sheep, poultry or horses), food source (purchased or mixed on the farm), water source (network, well or surface water), presence of standing water and reproductive disorders (repeat breeding, anoestrus, still birth, birth of weak calves, calving interval, abortion, number of calving and pregnancy, endometritis and retained fetal membrane). A bivariate analysis was performed to study the crude association between *Pestivirus* infection and the variables of

interest. For numeric variables, we used the Student's t-test. For categorical variables, we used the Chi-squared test, except when the sample size for any of the categories was small (i.e. lower than 5), in which case we used the Fisher's exact test. The association between *Pestivirus* infection (binary response) and the variables was further evaluated using a mixed-effects logistic regression model. To account for the two-stage sampling design, and therefore the lack of independence of samples, due to the fact that animals were grouped in herds, the herd was included in the model as a random effect.

Model building strategy: we started by including all the variables that complied with the inclusion criteria ($p<0.25$). To avoid the multicollinearity the Variance Inflation Factor (VIF) was evaluated. The fixed effect with high VIF were removed and the regression model was re-run. When two or more covariates had similar high VIFs, those with lowest significance in the univariate analysis were removed. The process was repeated until all variables had VIFs lower than or equal five (Dohoo et al., 2003). Then, one by one, variables were removed starting with the less significant, and the Aikake information criteria (AIC) was checked. If the AIC of the reduced model was lower, then the variable was permanently excluded, and we proceeded to eliminate the next less significant variable. Once all the remaining variables were significant, we computed the correlation matrix and excluded the variables that were strongly correlated (according to the criteria $rho>0.5$). Removal of the later variables caused some changes in the significance of the remaining ones, and further selection was needed, pursuing to obtain the lowest AIC. Finally, all the possible two-way interactions were evaluated, but none of them was significant. **There is no satisfactory test to evaluate the goodness of fit in the case categorical multilevel/hierarchical data (Perera et al., 2016). Therefore, we used the Hosmer-Lemeshow method combined with the estimation of the model accuracy.** ~~The validation of the model was performed with the Hosmer-Lemershow method for goodness of fit (Dohoo et al., 2003).~~ As the response is binary, the coefficients obtained are interpreted in

terms of odds ratio (OR). All statistical analyses were carried out using R statistical software (R core team, 2020).

2.4. Review of the presence of ruminant pestiviruses in North Africa

The review of the presence of ruminant pestiviruses in North Africa was performed using a systematic search and compilation methodology of peer-reviewed literature available in order to identify gaps of knowledge for future research. North Africa is the UN subregion comprised by Algeria, Egypt, Libya, Morocco, Sudan, Tunisia and Western Sahara. We searched Web of Science: All Databases (WoS; Thomson Reuters) literature database using "topic" searcher. We used the words "(*Pestivirus* AND Algeria OR Egypt OR Libya OR Morocco OR Sudan OR Tunisia OR Western Sahara)" (44 articles) and then we discarded research papers on Classical Swine Fever Virus (44 *Pestivirus* articles - 21 CSFV articles = 23 Ruminant *Pestivirus* articles from North-African countries). Finally, we added any relevant literature that was not originally included in WoS (2 articles).

3. Results

3.1. Serology, molecular characterization, and risk factors

The prevalence of antibodies against *Pestivirus* in cattle found in the present study, adjusted for the two-stage design, was 59.9% (138 out of 234) with a 95% CI [49.0-70.7%]. The overall seroprevalence of infected herds was 93.5% (29 out of 31) with a 95% CI [78.6%-99.2%] and the within-herd seroprevalence ranged from 0.0% to 100.0%. Considering the sensitivity and specificity of the IDEXX p80 test, 60% and 97%, respectively (Hanon et al.,

2017), and an estimated apparent prevalence of 59.9%, the true prevalence of disease would be 99.8%.

The model with all the factors that were significantly associated ($p<0.05$) with the presence of *Pestivirus* infection included: presence of sheep, size of herd, animal category, breed, presence of standing water and number of calving. After studying the correlation, the best-fitting model included: presence of sheep (OR=5.64; 95% CI [2.0, 15.9]; p=0.001) and animal category (cow, OR=3.80; 95% CI [1.6, 8.9]; p=0.002). The variance of the random effect was 0.48, therefore evidencing the heterogeneity among herds. **Model validation suggests that the model did not provide a good fit to the data ($p<0.001$), while the estimated model accuracy was only 60%.** RT-PCR resulted positive in 3 out of the 234 analysed animals. Only one of these three positive samples (a heifer of 20 months) could be sequenced targeting the *Pestivirus* 5'UTR region, confirming the presence of BVDV-1a in cattle from Algeria (Tiaret_2019; GenBank Acc. No. MT157227).

3.2. Review of ruminant pestiviruses in North Africa

Our literature review (25 research articles) confirmed the presence of ruminant pestiviruses in all North African countries, except in Libya and Western Sahara, where no data was available. A summary of the studies on *Pestivirus* in livestock in North Africa is presented in Table 1. The most studied and reported pestiviruses were *Pestivirus* A and B (BVDV-1, -2) in cattle and dromedary camels. However, the few studies on *Pestivirus* D (BDV) reported high seroprevalences in Algeria, Morocco and Tunisia, and an outbreak of severe clinical Border Disease in Tunisia in small ruminants. The review of the main risk factors for the presence of pestiviruses in livestock in North Africa found them to be heterogeneous (Table 1). On the other

hand, no information about *Pestivirus* in wild ruminants in North African countries was recorded.

4. Discussion

Ruminant pestiviruses are neglected pathogens in North Africa. However, the scarce data available suggest an endemic epidemiological scenario of *Pestivirus* in livestock. The antibody seroprevalence at population and farm levels obtained in the present study concur with the majority of the epidemiological data reported in cattle and dromedary camels in neighbouring North-African countries (Table 1). Surprisingly, the present study is in contrast to Derdour et al. (2017) that reported a very low prevalence of antibodies (1.4%) in cattle in Algeria, probably due to a sampling performed exclusively in intensive production systems, where the "hit and run" transmission strategy of *Pestivirus* (horizontal transmission between individuals) can be favored (Peterhans and Schweizer, 2010). The hypothesis of an endemic and heterogenous *Pestivirus* scenario in North-Africa is reinforced by the studies performed in small ruminants that showed the presence of a third *Pestivirus*, *Pestivirus* D (Border Disease Virus – BDV) in these species, with similar high antibody prevalences (17.7% to 68.2%) (Table 1). Additionally, the present study reported the first description of a BVDV-1a in North Africa, whereas BVDV-2a and BVDV-1b had been isolated from cattle in Tunisia (Thabti F et al., 2005b). Although the three PCR-positive animals could not be confirmed as persistently infected (i.e. two PCR-positive samples separated between 15 days), their presence together with the reported seroprevalence of antibodies in some farms, is highly suggestive of the presence of PI cattle in Algeria. Detection and elimination of PI individuals, and characterization of circulating viruses are cornerstones for eradication programs.

The risk factors detected in the present study (mixed herd [presence of sheep], and animal category [cow]) have been previously associated with *Pestivirus* infections worldwide (Schweizer and Peterhans, 2014). However, the specific risk factors for *Pestivirus* infection in ruminants in North Africa have not been analyzed in depth, and the few studies show a high heterogeneity of risk factors (Table 1), hindering the possibility of improving livestock production. In our study, the presence of sheep in the herd increased significantly the risk of *Pestivirus* infection (OR=5.65), which may be explained by the inter-specific infectious ability of pestiviruses, that facilitate their geographic dispersion and persistence in ruminant populations (Schweizer and Peterhans, 2014). Multi-species herds are a common practice in Algeria and even if there is a lack of information around their proportion in our study area, Mouffok (2014) reported 42% of mixed herds in the Northeastern part of the country. In our study, the proportion of herds of cattle mixed with sheep reached 48%. This practice allows a diversification of revenues for the farmer but should be avoided or at least cattle should be kept separated from other ruminants, in order to limit interspecific *Pestivirus* infection. Our results show that cows have a higher risk of *Pestivirus* infection as compared to heifers (OR=3.80), which coincides with previous studies (Schweizer and Peterhans, 2014; Selim et al., 2018), and is explained by the higher the age of cows that increases the probability of having been exposed to pestiviruses. We tried to minimize any selection bias in the herds and animals chosen, and therefore we consider the herds and animals selected were representative of the population. However, some sources of bias cannot be ruled out (e.g. if herds not officially registered are different from those registered, or if herds in which herd owners willing to participate are different from those which are not).

The role of wildlife as reservoir of pestiviruses has been proved in several wild species worldwide, being a risk factor for livestock. Pestiviruses A and D were reported to have a sylvatic cycle in white-tailed deer (*Odocoileus virginianus*; USA) and Pyrenean chamois

(*Rupicapra pyrenaica*; Spain) respectively (Fernández-Sirera et al., 2012; Passler et al., 2016). However, there is no research available on the presence of pestiviruses in North-African wildlife even though seven free-ranging wild ruminant species share territories with livestock like camels, dromedary and goat in North-African countries (IUCN, 2020). Additionally, *Pestivirus* D has produced high mortality rates in chamois, entailing a threat for its conservation (Fernández-Sirera et al., 2012). The conservation status of all North-African wild ruminants is of concern; two species being considered as critically endangered (Addax [*Addax nasomaculatus*], Dama Gazelle [*Nanger dama*]), one as endangered (Slender-horned Gazelle [*Gazella leptoceros*]), and four as vulnerable (Cuvier's Gazelle [*Gazella cuvieri*]; Nubian Ibex [*Capra nubiana*]; Aoudad [*Ammotragus lervia*]; Dorcas Gazelle [*Gazella dorcas*]). In that sense, *Pestivirus* circulation in domestic and wild ruminants should be of concern both for its economic impact but also from a wildlife conservation perspective.

5. *Conclusions*

In summary, pestiviruses are widespread in livestock in North Africa. However, there is a significant lack of both cross-sectional and longitudinal transboundary studies about the epidemiology and molecular variability of ruminant pestiviruses in livestock and wildlife in North Africa. This is of concern for livestock health and wildlife conservation, and needs to be addressed.

Declarations of interest

None

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Table 1. Results of studies investigating the seroprevalence of Pestiviruses in ruminants in North African countries

Country	Year of sampling	Pestivirus	Species	Diagnostic method	N	Prevalence	Risk factors	Ref.
Algeria	2011-2013	BVDV	Cattle	Ab-ELISA	360	1.4%	- Abortions	(Derdour et al., 2017)
	2019	BVDV-1a	Cattle	Ab-ELISA RT-qPCR	234	59.9% 1.3%	- Presence of sheep - Increase of age	Present Study
	2015-2016	BDV/BVDV	Sheep	Ab-ELISA VNT Ag-ELISA RT-PCR	576 197 689 689	68.2% 68.2% 0% 0%	- Climate: arid vs Mediterranean - Landscape: mountain vs plateau - Flock management: sedentary vs transhumant - Presence of goats	(Feknous et al., 2018)
	2016-2017	BVDV	Dromedary camel	Ab-ELISA Ag-ELISA	111	9.0% 41.4%	- Sheep, goat, cattle in mixed herd	(Saidi et al., 2018)
Morocco	1984	BVDV	Cattle	IFF	524	48.5%	- Extensive management system - Local ruminants - Ruminants without apparent respiratory symptoms	(Mahin et al., 1985)
	NA	BDV	Sheep	Ab-ELISA qPCR Ag-ELISA	760 543 150	28.9% 0% 0%	- Intensive farming - Presence of cattle	(Fassi et al., 2019)
	NA (1982?)	BVDV	Cattle	Disease	1			(Mahin et al., 1982)
	NA	BVDV	Cattle	Ab-ELISA	42	37.7%		(Lucchese et al., 2016)
Tunisia	1995	BDV	Sheep	Disease Sequencing	NA 9	NA	- Vaccine contamination	(Thabti et al., 2005a)
	1993	BDV	Sheep from 1 flock with BD clinical history	Abortion VNT	2,576 53 aborted sheep	17.7% 100%		(Zaeim et al., 1993)
	2001-2002	BVDV2a BVDV1b	Cattle from 2 farms (F1, F2) with BVD clinical history	Ab-ELISA PCR Sequencing	F1-188 F2-820 F1 F2	87% 82% 2.6% 0.2% BVDV2a BVDV1b	- Importation of infected cattle/semen	(Thabti et al., 2005b)
Egypt	NA	BVDV	Cattle Buffalo Sheep Goats Dromedary camel	VNT (BVDV strains)	128 150 178 137 59	49.2% 52.0% 27.5% 31.4% 52.5%	- Species: Cattle/Buffalo vs sheep/goat/dromedary	(Zaghawa, 1998)
	2011	BDV/BVDV	Sheep Goat	IHC MAbs RT-PCR MDBK Sequencing	5 4	0% 50% 0% 25% BVDV1b BVDV1a	-Neurological signs	(Abdel-Latif et al., 2013)
	2012-2013	BVDV	Cattle	Ab-ELISA	480	40%	- Species: Cattle vs Buffalo	(Selim et al., 2018)

			Buffalo		260	23%		
	2011	BVDV	Cattle Buffalo	Ab-ELISA Ag-ELISA MDBK IFAT IPMA	151 97 22 21 19 3	100% 62.2% 14.5% 13.9% 12.5% 1.9%	- NA	(El-Bagoury et al., 2012)
	2017	BVDV	Dromedary camel	Ab-ELISA Ag-ELISA RT-PCR	80 80 10	11.2% 7.5% 0%	Camels from Sudan	(El Bahgy et al., 2018)
Libya		No Data						
Western Sahara		No Data						
Sudan	2017	BVDV	Dromedary camel-smuggler into Egypt	Ab-ELISA Ag-ELISA RT-PCR	120 120 7	47.5% 31.6% 42.8%		(El Bahgy, et al., 2018)
	2000-2006	BVDV	Dromedary camel	Ab-ELISA Ag-ELISA RT-PCR	260 186 13	84.6% 7% 100%		(Intisar et al., 2010)
	2000-2012	BVDV	Dromedary camel	Ic-ELISA	474	9.0%	- Mixed virus infection - Pneumonia - Lacrimation	(Saeed et al., 2015)
	2005-2008	BVDV	Cattle	Ab-ELISA	688	25.7%	- Khartoum state - Rainy season (July to October) - Females - Old cattle - Abortions - Neonatal deaths	(Elhassan et al., 2011)

SUPPLEMENTARY MATERIAL

Table 1. Summary statistics on the study population.

Numerical

Variables	Min	Mean	Median	Max
Age (months)	6	59.5	50	156
Herd size	7	26.7	22	62

Categorical (no differentiation by herds)

Variables	Category	Nº of animals	Prevalence (%)
Animal category	Heifer	45	8.09
	Cow	190	50.21
Race	Monbéliard	49	8.94
	Holshtein	89	21.70
	Brune	1	0.43
	Fluck	1	0.00
	Mixed	94	27.23
Race	Pure	141	31.06
	Mixed	94	27.23

Categorical (differentiation by herds)

Variables	Category	Nº of herds	Nº of animals	Prevalence (%)
Food source	Mixed on the farm	8	73	17.02
	Purchased	6	47	14.47
	Both	16	115	26.81
Water source	Network	1	8	0.85
	Well or fodder	27	217	54.04
	Reserve on the surface	2	10	3.40
Mixture of other species	No	16	165	37.45
	Yes	14	70	20.85
Presence of sheep	No	5	46	5.53
	Yes	25	189	52.77
Presence of poultry	No	4	57	12.77
	Yes	26	178	45.53
Presence of horses	No	11	102	18.72
	Yes	19	133	39.57
Presence of cats	No	1	7	2.13
	Yes	29	228	56.17

Table 2. Results of the univariable analysis for all the potential risk factors studied, including odds ratios and p-values.

Variables	Level	Odds Ratio	CI 95%	p-value
Age		1.02	1.01-1.03	0.004
Animal category	Cows vs Heifers	3.89	1.61-9.40	0.003
Race	Mixed vs Pure	1.94	0.81-4.64	0.136
Gestation	Yes vs No	0.83	0.44-1.56	0.557
Stage of gestation		1.02	0.90-1.14	0.796
Number of calvings		1.27	1.10-1.48	0.002
Number of gestations		1.25	1.08-1.45	0.002
Number of calving	3 and 4 vs ≤ 2	1.50	0.69-3.24	0.306
	≥ 5 vs ≤ 2	2.91	1.29-6.58	0.010
Primipara-Pluripara	Primipara vs Heifer	1.09	0.38-3.15	0.876
	Pluripara vs Heifer	4.04	1.50-10.89	0.006
Herd size		0.99	0.95-1.02	0.451
Use of disinfectant	Yes vs No	1.28	0.51-3.24	0.603
Cleaning method	Both (sweeping and piping) vs Sweeping	0.84	0.32-2.19	0.720
	Not practice vs Sweeping	2.04	0.54-7.71	0.296
Mixture of other species	Yes vs No	2.18	0.87-5.45	0.096
Visit of another farmer	Yes vs No	1.24	0.39-3.98	0.712
Food source	Purchased vs Mixed on the farm	2.54	0.67-9.61	0.169
	Both (Mixed on the farm and Purchased) vs Mixed on the farm	1.15	0.39-3.33	0.803
Water source	Well vs Network	5.11	0.49-53.37	0.173
	Surface water vs Network	17.02	0.79-368.64	0.071
Quarantine	Yes vs No	1.79	0.49-6.50	0.376
Presence of calving box	Yes vs No	1.34	0.15-12.02	0.793
Brucellosis screening	Yes vs No	0.81	0.32-2.03	0.650
Tuberculosis screening	Yes vs No	0.81	0.32-2.03	0.650
Presence of sheep	Yes vs No	4.57	1.82-11.50	0.001
Presence of poultry	Yes vs No	2.02	0.57-7.21	0.277
Presence of horses	Yes vs No	3.28	1.48-7.30	0.004
Pasture practice	Yes vs No	0.37	0.07-1.89	0.234
Rivers and streams	Yes vs No	0.63	0.24-1.67	0.351
Standing water	Yes vs No	1.24	0.50-3.11	0.642
Artificial insult	Artificial vs Natural	1.32	0.15-11.83	0.806

Table 3. Model coefficients and their p-values for the final model. The variance of the random effect (herd) was 0.48.

Variables	Coefficients	p-values
Intercept	-1.96	0.001
Presence of sheep (yes vs no)	1.72	0.001
Animal category (cows vs heifers)	1.33	0.002