Research paper

Epidemiological study of the association between bovine gammaherpesvirus type 4 and reproductive disease in dairy cattle from northwestern Spain

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Abstract

Bovine gammaherpesvirus 4 (BoHV-4) has controversially been related with cattle reproductive disease. In the present study we analyze the relationship between exposure to BoHV-4 and reproductive performance in dairy cattle from northwestern Spain. A total of 2,022 sera from 50 farms were examined to detect anti-BoHV-4 antibodies. Herd and individual reproductive records were collected to analyze association with exposure to BoHV-4. In addition, 52 abortion cases were examined to detect BoHV-4 DNA. An individual seroprevalence of 66.6% and a herd prevalence of 98% were found. Exposure to BoHV-4 increased with age, particularly in individuals between 26-36 months old (OR = 2.7; CI 95%: 1.2-5.0, compared to animals < 26 months). Seroprevalence was not associated with herd fertility and herd abortion rate, but seropositive animals between 26-36 months presented prolonged calving to fertilizing insemination intervals (HR: 1.4; CI 95%: 1.2-2.0) as well as higher odds of an unsuccessful 1st insemination (OR: 2.5; CI 95%: 1.2-5.0). In abortion cases, BoHV-4 DNA was found in 12 vaginal swabs from 5 farms but not in any fetal tissue. Our results reveal an endemic, high and widespread exposure to BoHV-4 among dairy cattle from NW Spain with a limited impact in the reproductive performance of herds. The significantly worse reproductive performance of seropositive animals of 26-36 months of age may be the consequence of the establishment of primo-infections when moving heifers to lactation lots. Our findings may be useful to understand the potential population impact of BoHV-4.

Keywords: BoHV-4; fertility; abortion; seroprevalence; dairy cattle; Spain

1. Introduction

Bovine gammaherpesvirus 4 (BoHV-4) is a member of the subfamily *gammaherpesviridae,* which has controversially been related to cattle disease. This virus was firstly associated with respiratory disease and keratoconjunctivitis (Bartha et al., 1966), but it has been posteriorly detected in a wide variety of clinical signs (Thiry et al., 1989). In the past few decades, several reports have increasingly related this virus to different cattle reproductive diseases (reviewed by (Chastant-Maillard, 2015)). Thus, epidemiological investigations have suggested that BoHV-4 may play a role as an abortifacient (Czaplicki and Thiry, 1998) and the virus has also been detected in aborted fetuses and placental tissues (Cvetojević et al., 2016; Deim et al., 2007, 2006; Delooz et al., 2017). Moreover, BoHV-4 has been also suggested to be involved in uterine disease (Areda et al., 2018; Donofrio et al., 2009; Monge et al., 2006), acting as a co-factor for the establishment of metritis or endometritis in combination with other bacterial pathogens (Chastant-Maillard, 2015; Sheldon et al., 2009). Furthermore, the findings of a higher BoHV-4 seroprevalence in repeat breeders (Gür and Doğan, 2010) and reduced insemination success in BoHV-4 infected cows (Klamminger et al., 2017; Szenci et al., 2015) also suggest a possible role in cattle infertility.

However, there are also many uncertainties on the implication of BoHV-4 in those pathologies and on how it might cause disease. The virus has been detected in healthy animals (Luther et al., 1971; Belak and Palfi, 1974) and experimental reproductions of the infection have only occasionally elicited reproductive disease (Wellemans et al., 1986). Variations of virulence by genotype have been suggested as a possible explanation for these differences (Frazier et al., 2002), but this has not been proved up to date. Furthermore, some aspects of the epidemiology of BoHV-4 are still unclear and need a greater understanding. For example, serological dynamics in herds have been limitedly

explored (Díaz et al., 2019). Moreover, the epidemiological studies analyzing BoHV-4 in bovine reproductive disease have frequently been limited to specific clinical cases or carried out in a few farms, which makes it difficult to determine the population impact of the infection. Considering the potential secondary role of BoHV-4, determining its population impact might be especially interesting, since the health or economic consequences may gain relevance at the regional level, if the infection is widespread among herds. In this context, it is valuable to analyze the exposure to BoHV-4 in relation to different reproductive indicators, in order to acquire a better knowledge of the BoHV-4 epidemiology and assess its potential impacts. In addition, no large-scale studies have been carried out in Spain up to date, although this country accounts for the sixth highest cattle population in the European Union (EUROSTAT, 2020). Therefore, we have carried out this study with the aim to describe the exposure to BoHV-4 and analyze its relationship with the reproductive performance of dairy cattle.

2. Material and methods

2.1. Area, study design and sample and data collection

The study was conducted in Lugo, a province in northwestern Spain, which accounts for approximately the 20% of the dairy cattle production in the country (MAPA, 2019a). Husbandry is mainly intensive and the main breed is Holstein-Friesian. Around 75% of the herds in this area comprise less than 30 animals, and high dairy production levels are concentrated in a minority of farms that are usually enrolled in Health Defense Associations (HDA). Farms in HDA are under control programs for BVD, IBR and *Neospora caninum*. Briefly, these control programs include the serological control of the animals introduced in the herd and periodic samplings of bulk-tank and of a representative sample of animals to detect antigens and/or antibodies (depending on the pathogen). The

results of these tests are used to classify herds according to their sanitary status and determine the frequency of the next samplings (e.g. annual or infra-annual). Vaccination is allowed but it must allow the application of control strategies that differentiate vaccination from natural infection (in BVD or IBR). In addition, a compulsory control program for brucellosis is conducted in cattle farms from Spain and the region has been declared free of brucellosis (MAPA, 2019b). No control programs are carried out for other herpesviruses.

In 2015, we offered the participation in this study to dairy cattle farms enrolled in HDA from the region. Fifty of them were randomly selected to determine the seroprevalence to BoHV-4. Sample sizes were calculated for each farm according to the formula recommended by Thrusfield (2007), setting an estimated seroprevalence of 50% and a precision of 90%, which resulted in a total of 2,022 sampled animals. Blood samples were randomly collected from animals older than 6 months by venipuncture of the tail vein into a BD vacutainer® tube with no anticoagulant and kept refrigerated. Serum was extracted at the arrival at the laboratory by centrifuging the samples at 800 *g* for 5 min. Then, sera were stored at -20 ºC until analysis. The reproductive indicators of these farms were retrieved for a period of a year before sampling including: calving interval, calving to first service interval, number of artificial inseminations (AI) per pregnancy, first service pregnancy rate, calving to conception interval, fertility rate (defined as the annual mean of the proportion of eligible cows that were confirmed pregnant to insemination within a 21-day interval), percentage of abortions and percentage of heat detections. The average values of these indicators are summarized in Table 1. Whenever possible, individual information regarding the sampled animals was retrieved from the farms' records and comprised a period of one year prior to the sampling date. These data

consisted in age, number and dates of AI, presence/absence of gestation (confirmed by transrectal ultrasonography 30–40 days after AI) and calving to first service intervals.

In addition, bovine abortions submitted to our laboratory during a previous study in which we determine the presence of abortifacients in ruminants in the region between 2013-2015 (Díaz-Cao et al., 2018) were analyzed by PCR to detect the presence of BoHV-4 (52 abortion cases from 16 farms). Samples per aborted cow consisted in the brain, a pool of the fetal organs obtained during necropsies (lung, liver, kidney and spleen), the placenta and a vaginal swab collected after abortion. Organ pools were made by macerating 0.1 g of each organ in 16 mL of PBS using a mechanic homogenizer (Stomacher®, Seward, Worthing, UK) and vaginal swabs were eluted in 1 mL of PBS. The presence of other abortifacients in these cases was also analyzed by bacteriological culture and by qPCR for *Neospora caninum*, IBR, *Coxiella burnetii*, *Campylobacter* spp., *Salmonella enterica*, BVDV and *Leptospira* spp. (for further details, see Díaz-Cao et al., 2018).

2.2. Serological analysis, DNA extraction, PCR and sequencing

Serum samples were tested to detect antibodies against BoHV-4 using the commercial ELISA Kit: ELISA BOHV-4 ELISA Kit (Bio-X Diagnostics, Jemelle, Belgium). The procedure was performed following the manufacturer's instructions. Because cross-reactions with other herpesvirus might happen and Bovine alphaherpesvirus 1 (BoHV-1) is present in the area, we randomly selected, in each farm, ten samples already tested for BoHV-4 in 48/50 farms (n = 480 samples) and analyzed them to detect the presence of antibodies against BoHV-1. For this purpose, we used a commercial blocking gB ELISA: IBR gB X3 (IDDEX, Westbrook, ME, USA). Two farms were not analyzed because they reported vaccination against BoHV-1 in the last two years.

DNA extractions from the vaginal swabs, placentas and pools of organs from abortion cases were performed using the commercial kit Nucleospin Tissue (Macherey-Nagel, Düren, Germany). 200 µL of the pool of organs, 0.25 mg of the brain and the placenta and 200 µL of the vaginal swab elution were taken as the starting sample for the DNA extractions. When a vaginal swab tested positive to PCR, all the available organs of the aborted fetus (lung, liver, kidney and spleen) were also analyzed individually using 0.25 mg of sample for the extraction. The procedure was performed following the manufacturer's instructions.

We performed a nested PCR (nPCR) to detect BoHV-4 DNA by amplifying a sequence of 260 bp in the *orf* 20 gene. The protocol of this nPCR was originally developed by Egyed et al., (1996). We followed this protocol with the minor modifications described by Verna et al., (2012). Primers used for PCR were 5'-GTTGGGCGTCCTGTATGGTAGC-3' and 5'-ATGTATGCCCAAAACTTATAATATGACCAG-3' in the first round and 5'-TTGATAGTGCGTTGTTGGGATGTGGT-3' and 5'-CACTGCCCGGTGGGAAATAGCA-3' in the second nested round. The PCRs were run in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, California, USA) and the products were visualized in 1.5% agarose gels stained with Real-safe (Real Biotech Corporation, Taiwan).

One positive sample per PCR-positive farm was sequenced (BigDye Terminator v3.1 kit, Applied Biosystems, Foster City, California, USA) in the Sequencing and Fragment Analysis Unit of the University of Santiago de Compostela.

158 *2.3. Phylogenetic analysis*

159 An exhaustive search for all the sequences of the amplicon used in this study
160 deposited in GenBank was performed. When identical sequences came from the same
161 study, only one of them was included. Sequences were aligned using the program
162 ClustalX2 (Larkin et al., 2007). The selection of the nucleotide evolutionary model was
163 assessed with the software jModelTest 2.1.7 (Darriba et al., 2012). Phylogenetic trees
164 were inferred with RevBayes (Höhna et al., 2016) using a general time reversible model
165 with gamma distribution and proportion of invariable sites (GTR + Γ + I). Four Markov
166 chain-Monte Carlo (MCMC) chains were run for 100,000 generations with 10% of
167 burning and 10% of thinning. Mixing and convergence were assessed using the program
168 Tracer (Rambaut et al., 2013).

169 *2.4. Statistical analysis*

170 The association in the results obtained by the BoHV-4 and BoHV-1 ELISAs was
171 analyzed using the McNemar's test. The sera from farms sampled to determine
172 seroprevalence to BoHV-4 were classified into four groups according to the age of cows:
173 1: < 26 months; 2: 26-36 months; 3: 36-48 months and 4: > 48 months. The first cut-off
174 was set at 26 months old, since that was the average age at which heifers were transferred
175 to lactation lots. The remaining breaks were set following the average ages for subsequent
176 parturitions in the area based on local records. Differences in the individual
177 seroprevalence of BoHV-4 by age were analyzed by mixed logistic regression,
178 contrasting each category with the previous one. Spearman's correlation (ρ) was used to
179 analyze the relationships between herd seroprevalence and the values of the herd
180 reproductive indicators. The medians of abortion and fertility rates in the sampled herds
181 were used to classify herds into two groups (over and under median) for each variable
182 and differences in BoHV-4 seroprevalence between those groups were analyzed by mixed

logistic regression. The relationship between individual seroprevalence and the number of animals that achieved a gestation in the first AI was analyzed by mixed logistic regression in each age group. The herd was included as a random factor in all the mixed logistic regressions performed.

Cox regression models were performed to evaluate the existence of differences in the calving-fertilizing insemination interval regarding seropositivity to BoHV-4 by running separated models by age group (2, 3 and 4). Survival curves were determined using the Kaplan-Meier method.

The statistical analyses were performed using the software R (v. 3.5.2) (R Development Core Team, 2018) with the packages "lme4" for mixed models (Bates et al., 2015) and "survival" and "survminer" (Kassambara et al., 2019; Therneau et al., 2014) for the Cox regression and the survival analysis.

3. Results

A total of 1,347/2,022 animals tested seropositive for BoHV-4 (66.6%; CI 95% = 64.5-68.7) with a herd prevalence of 98% (49/50). The distribution of the within-herd seroprevalences is showed in Figure 1a and presented a mean of 67.6% and median of 80.0%. Individual seropositivity increased with age and statistically significant differences were found between groups 1 and 2 (OR = 2.7) and between groups 2 and 3 (OR = 1.9) (Figure 1b). In the subset of samples tested to detect both BoHV-4 and BoHV-1 antibodies, 318/480 (66.3%) resulted positive to BoHV-4 and 34/480 to BoHV-1 (7.1%). The 92.9% of BoHV-4 positives tested negative to BoHV-1 and no statistical associations ($p < 0.05$) were detected between the outcomes of both tests.

The calving interval and the number of AI per pregnancy presented a significant positive correlation with the herd seroprevalence of BoHV-4 ($\rho = 0.334$ and $\rho = 0.410$, respectively) (Table 1). However, no significant differences in individual seropositivity were found when comparing animals that came from herds with high fertility and abortion rates to those that came from herds with low rates ($p = 0.761$ and $p = 0.755$, respectively) (Table 2). A statistically significant association was found between individual seropositivity and 1st AI failure but only in 26-36 months-old animals ($p = 0.025$: OR: 2.5; CI 95%: 1.2-5.0) (Table 3). Similarly, the survival analysis also showed an association between seropositivity for BoHV-4 and prolonged calving-fertilizing intervals only for this age group ($p = 0.028$; HR: 1.4; CI 95%: 1.2-2.0) (Figure 2).

Five out of 16 farms with abortion problems were positive for BoHV-4. Fifty-two fetuses and vaginal swabs were analyzed. Analyzed fetal tissue tested negative but 12 vaginal swabs (23.1%) were positive for BoHV-4 by PCR. However, all the individual fetal organs which corresponded to these PCR-positive mothers tested negative. Four placentas out of 14 available tested positive to PCR (28.6%) and all of them corresponded to animals with PCR-positive vaginal swabs.

Five nPCR products were sequenced resulting identical between them and to other sequences isolated in different countries (Figure 3). The phylogenetic tree showed three main groups of strains, placing the European reference strain (Movar 33/63) and the American reference strain (DN 599) in different groups. The sequences isolated in this study were placed in Genotype 1 along with the European prototype.

4. Discussion

The potential population impact of BoHV-4 infection has not been extensively studied in the literature. In this context, we have conducted the first large-scale study of

229 the exposure to BoHV-4 in one of the most important regions for dairy cattle in Spain.
230 The performance of the ELISA test was good and no evidences of cross-reactions with
231 BoHV-1 antibodies were found. In addition, 31/50 farms did not present seropositive
232 results for IBR in representative samples of animals of 9-36 months old in the last two
233 years according to the records in the control programs. Thus, cross-reactions with BoHV-
234 1 are not expected to be an issue in our study. Our results reveal a very high and
235 widespread exposure of BoHV-4 (Figure 1a) that is among the previously reported values
236 (ranging from 4.2% to 98.2% (Ali et al., 2011; Metzler and Wyler, 1986)). Seropositivity
237 increased with age, especially in the 26-36 months group (Figure 1b), which is the
238 approximate time when heifers are transferred to lactation lots. We previously reported a
239 similar age pattern and high proportion of heifers seroconverting after their introduction
240 into lactation lots when we studied the serological dynamics of BoHV-4 in one herd,
241 (Díaz et al., 2019). Altogether, our results are consistent with the high incidence of new
242 infections that occurs when heifers are moved to lactation lots. The fact that 80% of the
243 animals older than 36 months tested seropositive and 50% of the herds presented
244 seroprevalences higher than 67.6% suggests a very efficient transmission of BoHV-4 in
245 these herds which easily leads to a high spread within the herd. Furthermore, the high
246 seroprevalence and proportion of positive herds, as well as the presence of young
247 seropositive animals, indicate an endemic infection in the herds of the region, which is
248 consistent with reports from other countries such as Belgium (Delooz et al., 2017), or
249 Turkey (Aslan et al., 2015) among others. This highlights the current need for a better
250 understanding of BoHV-4 infection to elucidate its real role and magnitude in cattle
251 disease.

252 Despite the high seroprevalences, BoHV-4 seropositivity was not associated with
253 herd fertility or abortion rates, thus indicating a low overall effect in these parameters. In

fact, both rates were good in some herds with high BoHV-4 seroprevalences, as showed in Figure 1a. The role of BoHV-4 in cattle fertility has scarcely been studied and results are difficult to compare due to their different approaches. Previous literature reported higher seropositivity in repeat breeders (Gür and Doğan, 2010) and in animals with prolonged calving to conception intervals (Kale et al., 2011). Likewise, infection was associated with a lower number of pregnant animals, prolonged first treatment to pregnancy intervals (Szenci et al., 2015) and reduced chances for insemination and pregnancy (Klamminger et al., 2017). In this study, we evaluated several commonly used reproductive indicators and found that herd seroprevalence was correlated with prolonged calving intervals and increased number of AI/pregnancy. Correlation analysis may be observed with caution since spurious relationships may occur. However, the lack of association between herd seroprevalence to BoHV-4 and abortion and fertility rates found in our study is highly suggestive of a low contribution of the virus to these parameters at herd level.

In this regard, when individual seropositivity was stratified by age, the multilevel analysis demonstrated a significant association with 1st AI failure only in 26-36 months-old animals. Likewise, a prolonged calving to conception intervals was also found in the survival analysis in this age group. It must be noted that a limitation of this survival analysis is that we cannot establish the exact moment of infection since we are detecting antibodies. However, the strong increase in seropositivity in animals of 26-36 years old (65% *vs.* 36% in animals < 26 months old; Figure 1b) suggests that most of infections in this age group are recent. BoHV-4 may affect fertility by having a negative effect as a co-factor on the endometrial immune response (Sheldon et al., 2009). However, this association has been controversial and Yang et al., (2019) showed a lack of association between subclinical endometritis and BoHV-4 latent or productive infection (primo-

infection or reactivation). Considering the age pattern of seropositive animals (Figure 1b), our results seem to suggest an effect of BoHV-4 on reproductive performance during primo-infections. An effect in a specific subpopulation of animals in the context of a high within-herd seropositivity would explain the lack of association that we found between BoHV-4 herd seropositivity and the herd-level fertility, since high proportions of seropositive animals with latent infections might mask the consequences of new infections. Unfortunately the validation of this hypothesis is beyond the possibilities of this study, but our results point out a new possible scenario to investigate the association of BoHV-4 with cattle disease.

Higher seropositivity of BoHV-4 and seroconversion in aborted cows have previously been reported (Czaplicki and Thiry, 1998; Delooz et al., 2017). Our results are not necessarily inconsistent with those studies because we could not evaluate individual performance, but they suggest an overall low contribution of BoHV-4 to cattle abortion. Consistently, we did not find BoHV-4 DNA in fetal tissue, but only in vaginal swabs, which is not sufficient evidence of the viral involvement in the outcome, as this sample may test positive in normally calved cows (Díaz et al., 2019). Furthermore, other potential abortifacients were detected in two out of the five positive farms (*Campylobacter* spp. and *Trueperella pyogenes*).

Our phylogenetic tree confirmed the detection of three genotypes in previous reports (Bellino et al., 2015; Gagnon et al., 2017; Verna et al., 2012). All our isolates and other strains of diverse geographical origin were placed into the proposed genotype 1 along with the European prototype (Movar 33/63). On the contrary, the other genotypes comprised strains of American origin. The variability of the strains has been suggested as one possible explanation for the diverse conditions in which BoHV-4 has been isolated (Frazier et al., 2002) and different *in vitro* kinetics depending on the strain have been

suggested (Morán et al., 2019). However, we could not explore this possibility as all our isolates were identical, which contrasts with the diversity of strains found in regions such as Argentina (Verna et al., 2012). This may be indicative of a geographical variation in the composition of strains and should be considered when comparing results from different regions.

In conclusion, our results show a high spread of BoHV-4 in dairy cattle from Northwestern Spain, but with a limited overall impact in reproductive performance, including fertility and abortion. However, the detection of a significantly worse performance in seropositive animals between 26 and 36 months of age suggests a potential relationship between BoHV-4 and the establishment of new infections and indicates a specific subpopulation in risk. Nevertheless, there are still questions to resolve regarding the pathogenic role of the virus and the ways it can cause disease. Establishing a relationship between within-herds dynamics of the infection and their effects on cattle reproductive disease may be a useful approach to unravel these questions.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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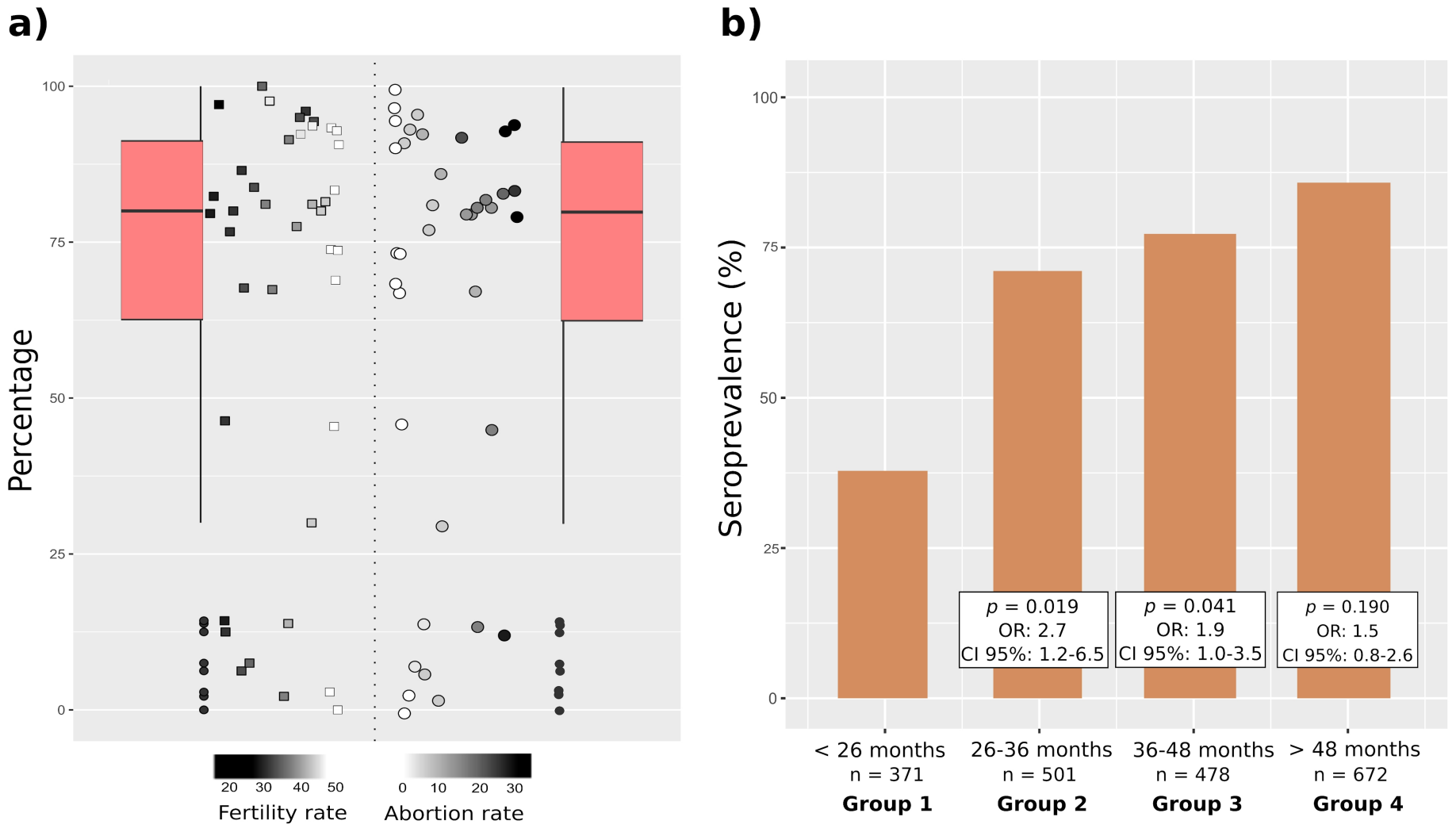
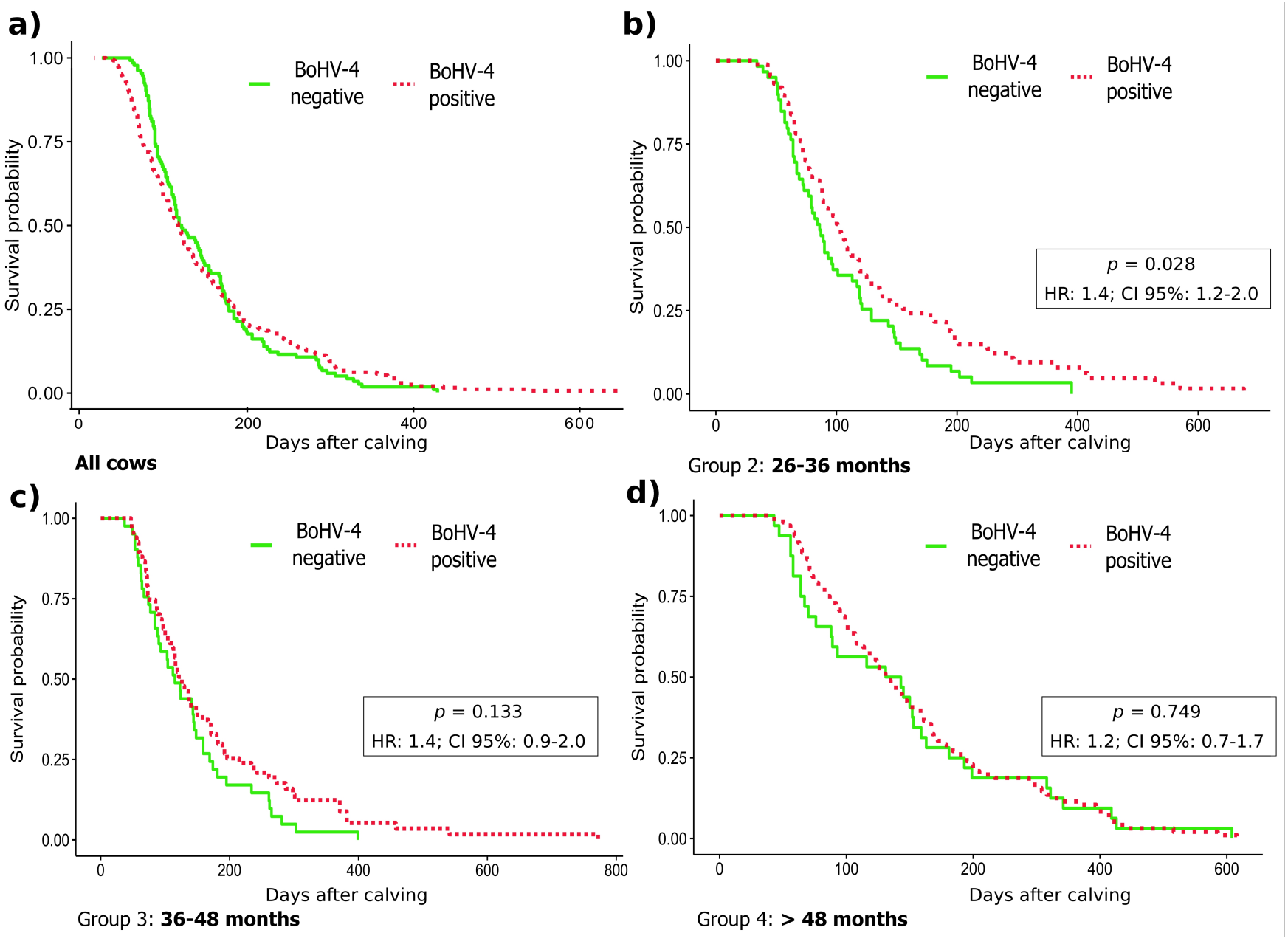
Figure legends

Figure 1. (a) Boxplot of the distribution of the within-herd seroprevalence of BoHV-4 combined with jitter plots of the distribution of herd-level fertility and abortion rate (n = 50 herds). (b) Distribution and statistical significance of the individual seroprevalence of BoHV-4 by age group.

Figure 2. Survival curves of the days needed to achieve pregnancy regarding seropositivity status to BoHV-4 in (a) all cows, (b) cows between 26-36 months, (c) cows between 36-48 months (d) and cows > 48 months.

Figure 3. Phylogenetic tree of BoHV-4 from this and others studies, obtained by the 50% majority rule. Branch lengths are proportional to the number of nucleotide substitutions per aligned positions (bar = 0.03). Numbers show posterior probabilities of branches and are displayed when probability > 70%.


a)
Percentage
100
75
50
25
0
20 30 40 50
Fertility rate
0 10 20 30
Abortion rate
b)
Seroprevalence (%)
100
75
50
25
0
p = 0.019
OR: 2.7
CI 95%: 1.2-6.5
p = 0.041
OR: 1.9
CI 95%: 1.0-3.5
p = 0.190
OR: 1.5
CI 95%: 0.8-2.6
< 26 months
n = 371
Group 1
26-36 months
n = 501
Group 2
36-48 months
n = 478
Group 3
> 48 months
n = 672
Group 4


a)
b)
c)
d)
BoHV-4 negative
BoHV-4 positive
Survival probability
Days after calving
All cows
Group 2: 26-36 months
Group 3: 36-48 months
Group 4: > 48 months
p = 0.028
HR: 1.4; CI 95%: 1.2-2.0
p = 0.133
HR: 1.4; CI 95%: 0.9-2.0
p = 0.749
HR: 1.2; CI 95%: 0.7-1.7

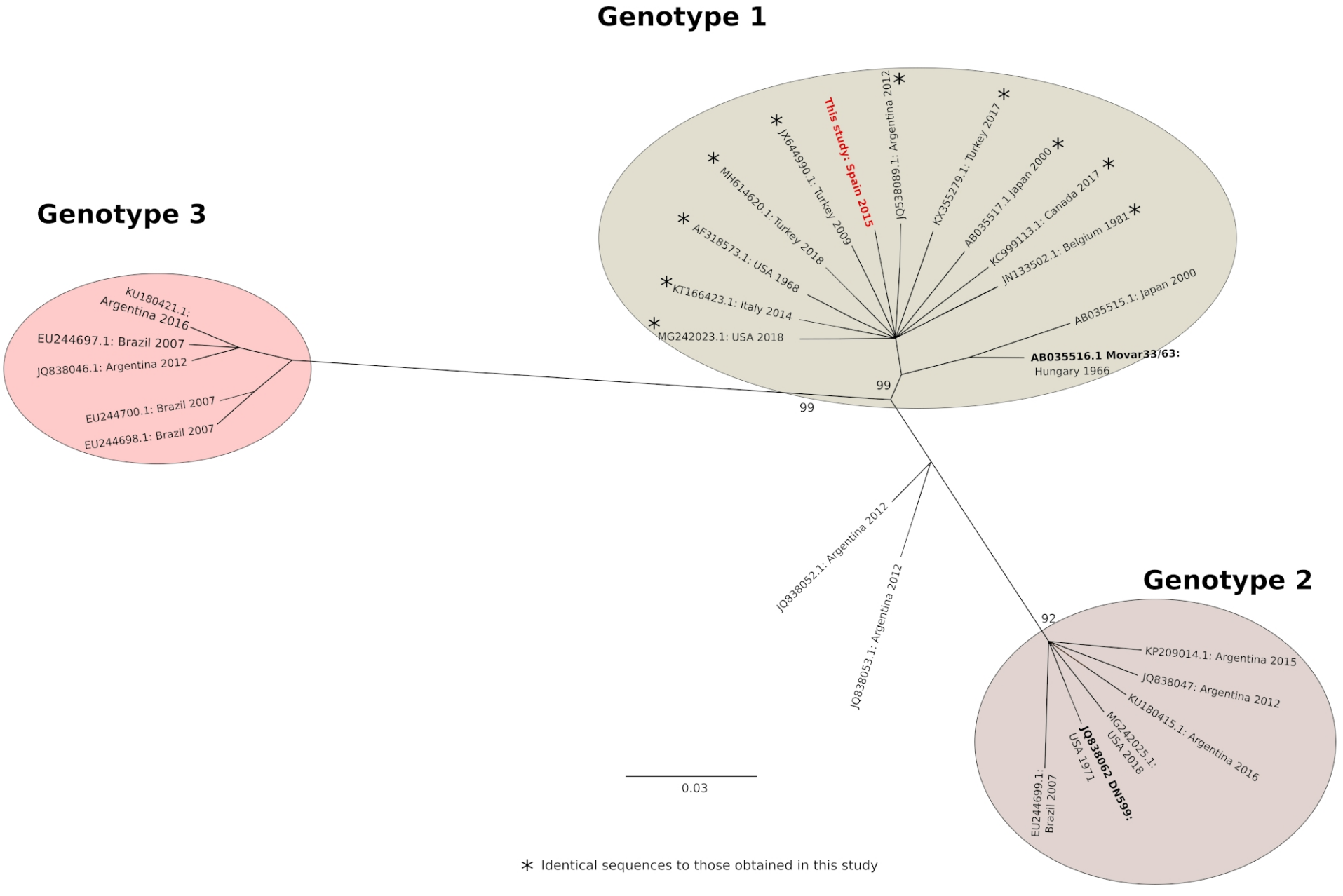

Genotype 1
Genotype 3
Genotype 2
This study: Spain 2015
JQ538089.1: Argentina 2012
KX355279.1: Turkey 2017
AB035517.1 Japan 2000
KC999113.1: Canada 2017
JN133502.1: Belgium 1981
AB035515.1: Japan 2000
AB035516.1 Movar33/63: Hungary 1966
JX644990.1: Turkey 2009
MH614620.1: Turkey 2018
AF318573.1: USA 1968
KT166423.1: Italy 2014
MG242023.1: USA 2018
99
99
KU180421.1: Argentina 2016
EU244697.1: Brazil 2007
JQ838046.1: Argentina 2012
EU244700.1: Brazil 2007
EU244698.1: Brazil 2007
JQ838052.1: Argentina 2012
JQ838053.1: Argentina 2012
92
KP209014.1: Argentina 2015
JQ838047: Argentina 2012
KU180415.1: Argentina 2016
MG242025.1: USA 2018
JQ838062 DN599: USA 1971
EU244699.1: Brazil 2007
0.03
∗ Identical sequences to those obtained in this study

Table 1. Summary results of reproductive indicators of the herds included in the study (n = 50) for the period of a year before sampling, and results of the Spearman's correlation (ρ) with the within-herd seroprevalence of BoHV-4

Parameter	Mean (standard deviation)	Median	Minimum-maximum	ρ with seroprevalence to BoHV-4 (*p*)
Calving interval (days)	427.0 (28.4)	424.0	388.0-510.7	0.334 (0.035)*
Calving to first service interval (days)	79.8 (9.1)	81	55.1-102.8	-0.176 (0.231)
No. AI per pregnancy	2.6 (0.6)	2.6	1.8-4.9	0.410 (0.027)*
First service pregnancy rate (%)	39.7 (10.6)	33.3	15.0-58.8	-0.213 (0.147)
Calving to conception interval (days)	149.1 (27.9)	148	103-236	0.153 (0.305)
Fertility rate (%)	33.9 (9.0)	34.8	15.4-51.7	-0.224 (0.127)
% Abortions	7.6 (8.1)	4.9	0.0-31.7	0.238 (0.145)
% Heat detection	52.7 (8.5)	53.9	35.1-67.0	-0.006 (0.966)

* statistically significant ($p < 0.05$)

Table 2. Differences in individual seropositivity for BoHV-4 according to the origin of the animals (herds with high or low fertility or abortion rates)

Parameter	Category[a] (parameter mean)	No. BoHV-4 positives/total	*p*	OR	CI 95%
Fertility rate	Low (25.6%)	613/969 (63.3%)	[b]		
	High (40.4%)	738/1053 (70.1%)	0.761	1.2	0.4-3.8
σ^{2} [c]	4.1				
Abortion rate	Low (2.3%)	509/767 (66.4%)	[b]		
	High (12.1%)	489/711 (68.8%)	0.755	1.2	0.3-4.5
σ^{2}	3.5				

[a] Cut-offs for high and low groups were set using medians: 34.8 for fertility rate and 4.9 for abortion rates

[b] Reference category

[c] σ^{2}: Variance of the random factor (herd)

Table 3. Results of the mixed logistic regression that evaluates the association between 1st AI failure and individual seropositivity for BoHV-4 for each group of age

Group of age	Category	No. of animals with an unsuccessful 1st AI/total (%)	*p*	OR	CI 95%	σ^{2} [a]
2: 26-36 months	Seropositive	212/304 (69.7%)	0.025	2.5	1.2-5.0	0.1
	Seronegative	88/176 (50.0%)	[b]			
3: 36-48 months	Seropositive	160/268 (59.7%)	0.676	0.8	0.3-2.0	0.2
	Seronegative	72/112 (64.3%)	[b]			
4: > 48 months	Seropositive	480/592 (81.1%)	0.340	1.4	0.7-3.3	0.3
	Seronegative	128/172 (74.4%)	[b]			

[a] σ^{2}: Variance of the random factor (herd)

[b] Reference category