Relationship between the prevalence of bovine viral diarrhea virus and animal welfare assessment, serum cortisol levels and hematological parameters in calves from three different Sicilian farms

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SUMMARY

Differences in management conditions between farms lead to different animal welfare levels between herds and contribute to the occurrence of some health disorders, including infectious disease. The aim of this study was to evaluate the relationship of prevalence of Bovine viral diarrhea virus (BVDV) and the changes in hematological parameters, serum cortisol concentration and welfare levels over the course of a year in calves of three farms from Sicily (Italy). For this purpose, 150 dairy Friesian calves in farm 1, 27 Limousine beef calves in farm 2 and 16 dairy Friesian calves in farm 3, aged 30±5 days, with body weight 40±6kg, were enrolled in the study. The welfare status of animals was assessed by the CReNBA. From each animal, blood samples were collected by coccygeal venipuncture at four time-point: in June (T1), in October (T2), in February (T3) and in June of the next year (T4). The serum cortisol concentration and the hematological parameters, including white blood cells (WBCs), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and platelets (PLT), were evaluated. No correlation between the prevalence of the infection and the CReNBA checklist was observed ($P>0.05$). A statistically significant change of the serum cortisol concentration and of hematologic parameters, except LYM and MCV, was observed in healthy calves from several three farms and in BVD infected calves from farm 2 and farm 3 throughout experimental period. According to the results gathered in the current study, it can be seen that the changes found in the investigated parameters were related to external and to internal factors in healthy calves and to direct or indirect action of the virus in BVD infected calves.

KEY WORDS

Bovine viral diarrhea; animal welfare; cortisol; hematological parameters; calves.

INTRODUCTION

Bovine viral diarrhea (BVD) is a disease of world interest, caused by a Pestivirus of the family Flaviviridae[1]. Two genotypes are known, BVDV1 and BVDV2[2] and a third genotype, known as a HoBi-like virus, is not yet official recognized, but clinical ad pathogenic features are similar to classic viral strains[3]. Bovine viral diarrhea had many clinical manifestations including: mild or subclinical infection (the most common form), persistent infection, mucosal disease (MD), and chronic BVD[4]. Observed clinical signs consisted of inappetence, lethargy, reduced milk yield, abortion[5]. Highly virulent strains of BVDV can produced lesions similar to those seen in cases of MD, such as severe and widespread ulceration of the oropharynx, larynx and esophagus and hemorrhagic enteritis[6]. Type 2 genotypes were generally considered more virulent but uncommon, causing, in some cases, severe disease, including thrombocytopenia. Worldwide attention is linked to its severe economic impairment, indeed, often remains sub-clinical and spreads rapidly on farms[7]. The virus can infect its natural host indefinitely with limited or no adaptive and innate immune response due to induced immunotolerance and active immunosuppression by the virus, respectively[8]. BVDV is divided into non-cytopathogenic (ncp) and cytopathogenic (cp) biotypes. Cytopathic effect can range from minor changes in the cell structure to cell dysfunction, cell lysis or cell transformation[9]. The ncp plays the major role in its effects on the host defenses by inhibiting various aspects of the innate immune system and creation of immunotolerance in the fetus during early gestation[5], before the

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fetus becomes immunocompetent[10]. Consequently, the fetus becomes immunotolerant to the virus and does not produce antibodies to it. At birth, persistently infected calves have constant viremia and serve as natural reservoirs of the virus[11]. Thus, they and will sustain viral replication and excretion for the rest of their lives[11]. Also, BVDV infection is established by inhalation, with viral replication initially in the oronasal mucosa and oropharyngeal lymphoid tissue. Viral replication and subsequent dissemination throughout the body continues in cells in lymphoid tissues, in circulating leucocytes in blood and in the bone marrow[12]. Viruses can result in bone marrow failure by one or more of three mechanisms: the direct inhibition or cytotoxicity of hematopoietic progenitor cells or marrow stromal cells required for hematopoiesis, or stimulated production of cytokines or of cytotoxic lymphocytes that inhibit the production or destroy hematopoietic cells[13].

Worldwide attention is linked to its severe impairment to animal health care and well-being[7]. Infectious diseases compromise hosts immune system and can affect several homeostatic function and well-being status[14]. Nowadays, the interest in farm animal welfare status is increasing. In particular, for farmed animals, such as cattle, health and biological functioning are often prioritized. Despite the action of homeostatic mechanisms to maintain blood parameters within physiologic levels, changes in metabolites and hormones occur during peculiar life phases of farm animals[15-18]. The level of animal welfare in the three farms has been calculated through the application of the CReNBA checklist. The Welfare Quality protocols of calves is divided into four essential principles of welfare: good feeding, good housing, good health and appropriate behavior[19]. Differences in management conditions between farms lead to different welfare levels between herds[20] and contribute to the occurrence of some health disorders, including infectious disease[21].

The aim of this study was to evaluate the relationship of prevalence of BVDV and the changes in hematological parameters, serum cortisol concentration and welfare levels over the course of a year in calves of three farms from Sicily.

MATERIALS AND METHODS

Animal and experimental design

The study was carried out on three calves' farms, located in Ragusa, Sicily, Italy (latitude 36°55'45"48 N, longitude 14°43'4"80 E, altitude 540mt above sea level). A total of 193 beef calves (150 dairy Friesian calves in farm 1, 27 Limousine beef calves in farm 2 and 16 dairy Friesian calves in farm 3), aged 30±5 days, with body weight 40±6kg, were enrolled in the study.

On all farms, calves were housed in barns with access to a grazing area at least 10 hours a day. They were fed a balanced diet daily (fodder, hay and silage) and water was available *ad libitum*.

All calves were subjected to a clinical exam and were controlled daily for clinical signs of disease. At enrollment animals were tested for bovine viral diarrhea (BVD) disease. Different prevalence of infection was found among the farms with 0.7% of prevalence of infection in farm 1 (1 positive calves out of 150 calves), 18.5% of prevalence of infection in farm 2 (5 positive calves out of 27 calves) and 12.5% of prevalence of infection in farm 3 (2 two positive calves out of 16 calves).

The CReNBA method was used for the assessment of the quality of animal welfare maintained on each farm. The checklist provides a 360° view of the farm by the scores assigned to explicit criteria. The evaluators are qualified veterinarians who take a specific training course. Structural, managerial and biosecurity aspects contribute to determining the final score of the production enterprise, together with the evidence of the animals' ability to adapt to the environment. The checklist is composed of five areas of investigation: Area A "Farm management and personnel", Area B "Facilities and equipment", Area C "Animal-based measures" for carrying out the assessment of the risk and of the consequent negative effects on calves, Area D "Biosecurity", Area E "Inspection of microclimatic environmental conditions and alarm systems" in the event of serious negative events (e.g. fire), for a total of 90 items. The score for each item was put into the appropriate database created by CReNBA (http://benessereruminanti.izsler.it), and then a score for each macro-area and an overall score for the farm was obtained. Certification of "Animal Welfare and Biosecurity Assessment" inspected status by CReNBA is the end of the procedure. The score ranges from 0 to 100 and identifies general welfare conditions of the herd, bracketing scores into "unclassified", "acceptable", "enhanced" and "excellent" categories. The protocol of this study was carried out in accordance with the standards recommended by the *Guide for the Care and Use of Laboratory Animals* and Directive 2010/63/EU.

Blood sampling and laboratory analysis

Throughout one year, from each animal, blood samples were collected in June (T1), October (T2), February (T3) and in June of next year (T4). From each animal, two blood samples were collected by means of coccygeal venipuncture into one vacutainer tube containing EDTA and in into one tube with clot activator. The blood samples were placed in refrigerated bags and transported to the laboratory for analysis. Upon arrival to the laboratory, EDTA whole blood samples were processed within 2 hours by means of an automated hematology analyzer (HeCo Vet C; SEAC, Florence, Italy) for the evaluation of complete blood count including white blood cells (WBCs), red blood cells (RBCs), hematocrit (Hct), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and platelets (PLTs). Leukocyte identification and counting was performed on all whole blood samples by manual analysis. Specifically, two peripheral blood smears were performed for each sample and, after air drying, the obtained blood smears were stained through Dif-Stain kit (Titolchimica srl, Rome, Italy). The same laboratory professional has later performed the microscopic analysis of blood smears by using an optical microscope (Nikon Eclipse e200; Nikon Instruments Europe BV, Amsterdam, The Netherlands). A manual 200-cell differential count was performed on each blood smear. For each animal, the leukocyte differential count was calculated by averaging of the data recorded from each blood smear of the same sample. The blood samples collected into tube with clot activator were allowed to clot overnight at 4°C before centrifuged at 1000g for 20 minutes at 2-8°C. The obtained sera were analyzed to assess the concentration of cortisol using an ELISA kit specific for ovine species (Cortisol ELISA kit, Elabscience Biotechnology Inc. Kampenhout, Belgium) by means of a microwell plate reader (Sirio, SEAC, Florence, Italy). All calibrators and samples were run in duplicate and samples exhibit-

ed parallel displacement to the standard curve for both ELISA analyses. Both the intra- and the inter-assay coefficients of variation were of <10%.*2.3*

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The obtained data were analyzed for normal distribution by Kolmogorov-Smirnov test. Data obtained from healthy calves of each farm and from BVD infected calves from farms 2 and 3 resulted normally distributed (P>0.05,) and parametric analysis was applied to assess the influence of sampling time. In particular, one-way analysis of variance (ANOVA) for repeated measure was applied and Bonferroni test was performed for post hoc comparison. Data obtained from BVD infected calves from farm 1 did not pass the normality test (P<0.05) and Kruskal-Wallis test followed by Dunn's multiple comparison test were applied to assess the effect of sampling time. P<0.05 was considered statistically significant. The correlation between BVD prevalence of infection in three farms and animal welfare assessment areas was studied by Pearson's correlation test. Data were analyzed using statistical software Prism v.5.00 (Graphpad Software Ldt, USA, 2003).

RESULTS

All three tested farms reached an overall animal welfare score greater than 60%, the specific results of welfare assessment Areas in the three farms are showed in Table 1. The prevalence of BVD infection resulted no correlated with animal welfare

Table 1 - Mean values ± standard deviation (±SD) of hematological parameters obtained at June (T1), October (T2), February (T3) and June of next year (T4) in healthy calves of the three farms (farm1, farm2 and farm3). *Significant effect of time (P<0.05):* [a]vs T1; [b]vs T2; [c]vs T3; [d]vs T4.

Hematological Parameters	Farms	Experimental period			
		T1	T2	T3	T4
RBC *(10^6/µl)*	1	5.97±0.6	6.66±0.2[a,c,d]	6.15±0.5	5.85±0.4
	2	6.77±0.8	6.23±0.7	5.87±0.46	5.97±0.7
	3	5.12±0.9	5.00±0.6[c]	5.99±0.5	5.64±0.8
HGB *(g/dl)*	1	9.97±1.4	11.27±0.5	10.38±0.6	10.55±0.8
	2	11.43±1.1[c,d]	11.2±0.8[c]	10.31±0.4	10.15±0.7
	3	10.01±1.3	10.36±0.9	11.89±0.5	10.70±1.1
HCT *(%)*	1	24.48±3.6	25.45±1.2	23.92±1.5	23.48±1.9
	2	28.18±2.6[b,c,d]	25.02±1.9	23.60±0.9	24.95±1.3
	3	22.93±3.6	22.27±2.3	27.66±1.4[a,b]	24.49±2.7
MCV *(fl)*	1	41.05±4.1	38.28±2.2	38.93±1.5	40.15±2.1
	2	41.92±3.9	40.38±3.1	40.42±2.6	43.08±1.4
	3	45.1±2.8	44.74±2.4	46.34±2.3	43.60±1.6
MCH *(pg)*	1	16.72±1.7[d]	16.93±0.8	16.9±0.7	18.07±0.9
	2	17±1.6	18.08±1.6	17.62±1.2	17.13±0.7
	3	19.8±1.7	20.84±1.4	19.94±1.2	19.07±0.9
MCHC *(g/dl)*	1	40.7±0.7[b,c,d]	44.25±0.9	43.40±0.9[d]	44.98±0.2
	2	40.53±0.3[b,c]	44.78±0.9	43.58±0.8	39.73±0.9[b,c]
	3	43.86±2.4	45.97±2.5[c]	43.00±0.7	43.67±0.8
PLT *(10^3/µl)*	1	321±112.91	161.31±91.0[d]	225.5±77.4	366.79±141.8
	2	197.78±112.6	269.85±140.9	272.83±124.4	217.5±98.4
	3	130.93±94.5	159.41±76.1	228.30±98.4	217.00±52.9
WBC *(10^3/µl)*	1	6.85±1.2	6.42±1.1	6.74±0.9	8.72±3.0
	2	8.86±2.3	6.97±1.8	6.96±1.6	6.31±1.3
	3	4.41±3.1[b]	8.46±2.7	6.72±1.3	6.51±2.5
NEU *(10^3/µl)*	1	2.57±0.8	2.27±0.6	2.41±1.2	4.95±3.2
	2	2.79±0.7	3.13±0.9	2.47±0.9	1.58±0.8
	3	0.84±0.6[b,c,d]	1.86±0.8	2.12±0.7	1.90±0.4
LYM *(10^3/µl)*	1	3.15±1.8	2.51±1.3	2.99±1.7	2.49±0.8
	2	4.82±2.6	2.59±0.9	2.96±0.5	3.63±1.6
	3	2.60±3.1	5.39±2.2	3.14±1.4	3.24±2.2
MONO *(10^3/µl)*	1	0.43±0.1[c,d]	0.65±0.1	0.85±0.3	0.75±0.2
	2	0.69±0.1	0.63±0.1	0.71±0.2	0.59±0.1
	3	0.30±0.2	0.51±0.2	0.53±0.3	0.45±0.2
EOS *(10^3/µl)*	1	0.63±0.3	0.78±0.3[c]	0.32±0.2	0.43±0.3
	2	0.47±0.41	0.48±0.2	0.72±0.2	0.42±0.4
	3	0.64±0.5	0.62±0.6	0.84±0.3	0.83±0.7
BASO *(10^3/µl)*	1	0.06±0.1[b,c]	0.19±0.1[d]	0.16±0.1	0.09±0.1
	2	0.09±0.1	0.15±0.1	0.10±0.1	0.09±0.1
	3	0.03±0.1	0.08±0.1	0.16±0.2	0.09±0.1

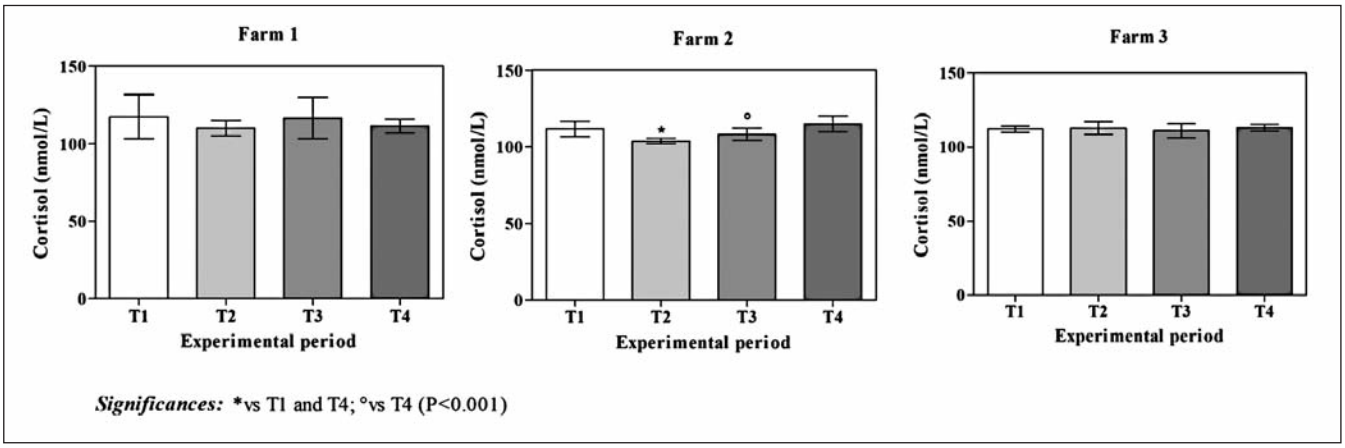

Figure 1 - Mean values ± SD of serum cortisol obtained in June (T1), October (T2), February (T3) and June (T4) in healthy calves of the three farms.

Table 2 - Mean values ± standard deviation (±SD) of hematological parameters obtained at June (T1), October (T2), February (T3) and June of next year (T4) in BVD infected calves of the three farms (farm1, farm2 and farm3). *Significant effect of time (P<0.05):* [a]vs T4; [b]vs T2; [c] vsT1.

Hematological Parameters	Farms	Experimental period			
		T1	T2	T3	T4
RBC *(10^6/µl)*	1	6.45	6.45	7.06	7.65
	2	5.86±1.1	5.03±1.1[a]	5.25±0.9	5.97±0.7
	3	5.02±1.8	5.05±0.8	4.15±0.5	3.99±1.0
HGB *(g/dl)*	1	9.87	9.87	9.61	13.4
	2	10.34±1.2	9.44±1.3	9.68±0.9	10.15±0.7
	3	10.48±1.58	10.00±0.9	9.2±0.1	9.08±0.6
HCT *(%)*	1	21.9	21.9	21.3	31.3
	2	25.48±4.2[b]	21.04±3.8	21.50±2.2	24.95±1.3
	3	22.40±7.5	22.05±2.5	20.00±0.4	19.85±0.9
MCV *(fl)*	1	34	34	30.2	40.9
	2	43.74±3.4	42.36±4.3	41.42±3.6	43.08±1.4
	3	44.75±1.1	44.00±2.3	48.65±4.3	48.50±3.8
MCH *(pg)*	1	15.3	15.3	13.6	17.6
	2	17.84±1.6	19.12±2.2[a]	18.68±1.7	17.13±0.7
	3	21.65±4.6	19.95±1.2	22.40±2.3	22.25±1.7
MCHC *(g/dl)*	1	45	45	45.1	43
	2	40.8±1.5[b]	45.14±2.7[a]	44.50±1.4[a,c]	39.73±0.9
	3	48.30±9.2	45.40±0.4	46.10±0.6	45.95±0.02
PLT *(10^3/µl)*	1	51.6	51.6	129	21.1
	2	239.8±62.2	338.80±125.7	348.60±155.7	217.50±98.4
	3	214.50±14.8	205.50±21.9	181.50±55.9	181.35±56.4
WBC *(10^3/µl)*	1	16.5	16.5	16.5	14.1
	2	6.43±1.7	6.57±1.4	6.99±0.9	6.31±1.3
	3	4.35±1.0	7.63±0.1	8.23±0.7	8.07±0.2
NEU *(10^3/µl)*	1	2.87	2.87	1.79	12.2
	2	3.43±1.6	3.18±1.0	3.00±1.6	1.58±0.8
	3	1.25±0.3	1.26±0.7	1.61±0.2	1.45±0.7
LYM *(10^3/µl)*	1	12.5	12.5	12.7	11.35
	2	1.64±0.6	1.86±1.04	2.51±1.1	3.63±1.6
	3	2.01±1.4	4.18±1.9	4.17±1.9	4.02±1.4
MONO *(10^3/µl)*	1	0.65	0.65	1.39	0.49
	2	0.58±0.2	0.91±0.7	0.75±0.1	0.59±0.1
	3	0.44±0.1	0.46±0.2	0.55±0.1	0.70±0.1
EOS *(10^3/µl)*	1	0.08	0.08	0.37	0.002
	2	0.73±0.3	0.52±0.5	0.63±0.7	0.42±0.4
	3	0.56±0.1	0.59±0.3	0.77±0.5	0.61±0.1
BASO *(10^3/µl)*	1	0.41	0.41	0.21	0.07
	2	0.05±0.1	0.10±0.1	0.24±0.4	0.09±0.1
	3	0.09±0.1	0.09±0.1	0.08±0.1	0.20±0.2

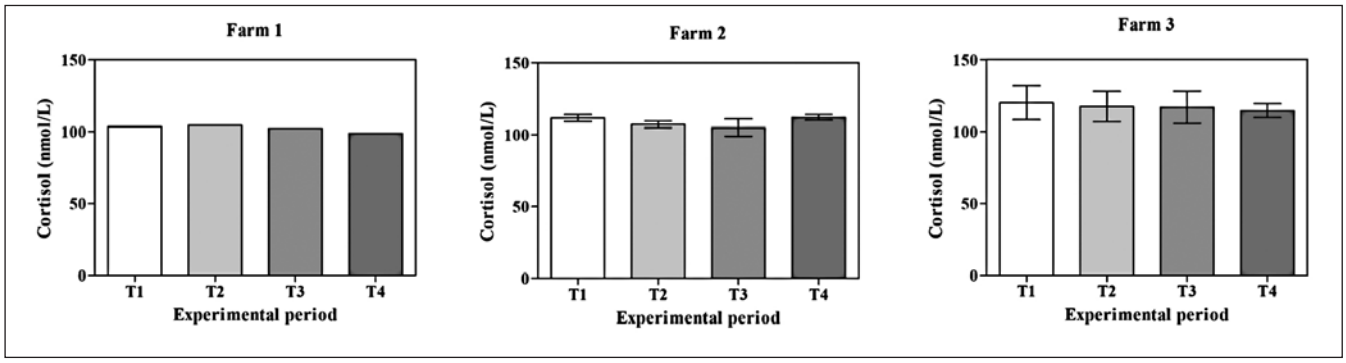

Figure 2 - Mean values ± SD of serum cortisol obtained in June (T1), October (T2), February (T3) and June (T4) in BVD infected calves of the three farms.

assessment areas (P>0.05). According to the statistical analysis of data serum cortisol showed unchanged values in healthy calves of farm 1 and farm 3 throughout the study period (P>0.05), whereas, the healthy calves from farm 2 showed lower cortisol values at T2 than T1 and T4 (P<0.001) and higher values at T4 than T3 (P<0.001) (Figure 1). Serum cortisol concentration showed unchanged values in BVD infected calves from all three farms (Figure 2). In Tables 1 and 2 the mean values of hematological parameters obtained from healthy and BVD infected calves throughout experimental period are reported, respectively. The WBC parameters showed no statistically significant values in healthy calves of farm 1 and farm 2 and in BVD infected calves in all studied farms (P>0.05). The NEU parameter values showed unchanged values in healthy calves of farm 1 and farm 2 and in BVD infected calves in all three studied farms (P>0.05), whereas, the healthy calves from farm 3 showed lower values at T1 than T2, T3 and T4 (P<0.001). No statistically significant changes in LYM values were observed in healthy and BVD infected calves from all the three studied farms throughout the study period (P>0.05). Healthy calves from farm 1 showed lower MONO values at T1 than T3 and T4 (P<0.001). The RBC values were higher in healthy calves from farm 1 at T2 than T1, T3 and T4 (P<0.001) and in healthy calves from farm at T3 than T2 (P<0.001). Regarding BVD infected calves from farm 2, RBC parameters showed higher values at T4 respect to T2 (P<0.05). The HGB values were higher in healthy calves from farm 2 at T1 than T3 and T4 and at T2 than T3 (P<0.001) and from farm 3 at T3 respect to T1 and T2 (P<0.001). The healthy calves from farm 2 showed higher HCT values at T1 than T2, T3 and T4 and at T4 than T3 (P<0.001) whereas the healthy calves from farm 3 showed higher values at T3 respect to T1 and T2 (P<0.001). Regarding BVD infected calves of farm 2, HCT values showed higher values at T1 than T2 (P<0.05). The MCV values showed unchanged values in healthy and BVD infected calves from all the three studied farms throughout the experimental period (P>0.05). Higher MCH values were found in healthy calves from farm 1 at T4 than T1 (P<0.001) and in BVD infected calves from farm 2 at T2 respect to T4 (P<0.001). The MCHC showed lower values in healthy calves from farm 1 showed lower values at T1 than T2, T3, T4 and at T3 respect to T4 (P<0.001), in healthy calves from farm 2 at T1 respect to T2 and T3, and at T4 than T2 and T3 (P<0.001), in healthy calves from farm 3 at T3 than T2 (P<0.001). Regarding BVD infected calves of farm 2, MCHC values showed higher values at T2 and T3 respect to T1 and T4 (P<0.001). Healthy calves from farm 1 showed higher PLT values at T4 than T2 (P<0.01).

DISCUSSION

According to the results of the current study the overall animal welfare score was greater than 60% in the three farms under investigation and a low prevalence of BVD infection was observed. The CReNBA's checklist represents a functional, reproducible, impartial and smart tool based on risk analysis and provides a numerical index of animal welfare, based on the data collected in each farm[22]. A low CReNBA checklist score may be highly correlated with a higher incidence of disease development[23]. Studies reported that management practices and housing system commonly influenced animal well-being and their comfort[24] and the biosecurity was useful to prevent the introduction of a disease agent to herds[25]. A good management and more controls on farms should reduce the spread of disease and increase well-being score of each animal. In this study, in healthy calves, cortisol values were higher than the values reported by Doornenbal et al.[26] (73±05 nmol/L). Although there was no correlation between prevalence of infection and evaluation of CReNBA checklist, the animal well-being and the factor stress were closely linked. In particular, higher cortisol concentration was observed in farm with lower overall score of animal's well-being. In healthy animals, the increase in cortisol values and the change in hematological parameters were expected for several factors, including physiological stage[27], the productive attitude (beef, dairy calves) of the animal[28], early separation of cow and calf[29], painful procedures (dehorning, branding), social environment (overcrowding or isolation), transport and bad management conditions[30]. In unhealthy animals, no significant changes in serum cortisol concentration were observed. Which is in line with the result by Ganheim et al.[30], which explained this as a low toxicity of the virus or the suboptimal time of blood sampling for detection of this parameter. BVDV infection can cause destruction of immunocompetent cells and impairs function of surviving cells. In acute infection, BVDV produce mild lymphopenia and no thrombocytopenia[10]. Subsequently, peripheral blood neutrophil, lymphocyte, and platelet counts decreased[31] as a result of cytotoxic products induced by virus[32]. Also, lymphopenia from viral infection may be partially due to stress of disease, caus-

ing increased blood cortisol levels. In unhealthy animals, we have obtained statistically significant variations of RBC, HCT and MCHC in farm 2. Other studies observed an affinity for the cells of lymphoid tissue, often lymphopenia and neutropenia, and also thrombocytopenia[5; 33]. This could be due to the viral infection altering immune system with suppression of innate immune functions[34].

CONCLUSION

The results gathered in the current study confirm that the application of a validate checklist can be a fundamental tool for veterinarians to detect stress condition in livestock. The high levels of biosecurity and good farm management could have a positive effect on well-being level, on the health care and, consequently, on the prevention of infectious disease. Healthy calves showed significant changes in the values of the hematological parameters that could be related to many factors as environmental conditions, management, feeding, housing, physiological state of the calves and the variable farm's well-being scores. Moreover, the changes of hematological parameters observed in unhealthy animals could be probably due to the direct or indirect actions of the virus. Prophylactic measures, linked to biosecurity, such as routine diagnosis, reproductive control, acquiring animals of secure provenience, should be recommended in order to reduce and/or control the BVD infection spread.

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