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Haptoglobin and its association with naturally occurring diseases in Holstein heifer calves

[*Haptoglobina e sua associação com doenças ocorridas naturalmente em bezerras da raça Holandesa*]

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ABSTRACT

The present study evaluated the use of haptoglobin (Hp) as an indicator of health and performance in 166 Holstein heifer calves reared in an intensive production system. Calves were evaluated at D6-9; D10-13; D20-23; D35-38 and D65-68, corresponding to the days of life. The absence or presence of diseases was evaluated by physical examination and classification of scores. The performance parameters evaluated were body weight, height at withers and hind width. Hp was measured by spectrophotometric technique. The highest prevalence of diarrhea (59.4%; 98/165) was observed in D10-13, bovine respiratory disease (BRD) was on D35-38 (25.8%; 42/163), and umbilical inflammations in D6-D9 (7.8%; 13/166). Highest values of Hp were observed in animals with diarrhea (P=0.02), and umbilical inflammation (P=0.057), in comparison with the group of healthy calves. A significant negative correlation was observed between Hp and performance index. This protein presented an important relation with diarrhea and performance of the calves, opening perspectives on its utilization as a biomarker of diseases.

Keywords: diarrhea, respiratory disease, inflammation, acute phase proteins

RESUMO

O presente estudo avaliou o uso da haptoglobina (Hp) como indicadora de sanidade e desempenho em 166 bezerras Holandesas criadas em um sistema de produção intensivo. As bezerras foram avaliadas nos momentos D6-9; D10-13; D20-23; D35-38 e D65-68, sendo estes correspondentes aos dias de vida. A ausência ou a presença de doenças foi avaliada por meio do exame físico e da classificação por escores. Os parâmetros de desempenho avaliados foram peso corporal, altura de cernelha e largura de garupa. A Hp foi mensurada por técnica espectrofotométrica. A maior prevalência de diarreia (59,4%; 98/165) foi observada em D10-13, doença respiratória bovina (DRB) ocorreu em D35-38 (25,8%; 42/163) e inflamações umbilicais em D6-D9 (7,8%; 13/166). O valor de Hp foi maior nos animais que apresentaram diarreia (P=0,02) e inflamações umbilicais (P=0,057), em comparação ao grupo de bezerras saudáveis. Houve correlação negativa significativa entre a Hp e os índices de desempenho. Essa proteína apresentou uma importante relação com a diarreia e com o desempenho das bezerras, abrindo perspectivas sobre a sua utilização como biomarcadora de doenças.

Palavras-chave: diarreia, doença respiratória, inflamação, proteína de fase aguda

INTRODUCTION

Immune system of calves gradually matures after birth through repeated contact with the antigens, since the sterile uterine environment and synepitheliochorial placenta make them agamoglobulinemic and dependent on maternal colostrum (Chase *et al*., 2008), thus increasing their vulnerability to frequent illnesses in this period of life. Diarrhea affects calves more significantly between seven and fourteen days of life, with prevalence rates from 68.9 to 92.8% (Novo *et al*., 2017; Martin, 2017). Bovine Respiratory Disease (BRD) is the main cause of

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morbidity and mortality in calves from 60 days to weaning, with prevalence of around 14% (Lago *et al*., 2006). The occurrences of diseases are directly impacted by treatment costs and deaths, and indirectly impacted by the decrease in weight gain and performance of calves (Baptista *et al*., 2017).

Much research has been carried out to improve early detection methods or even to predict the risk of disease in order to prevent direct and indirect losses. Diarrhea can be detected with the use of scoring systems, clinical signs and body temperature (Deignan *et al*., 2000). For the diagnosis of BRD, different methodologies were used in suckling calves, such as chest ultrasound (Buczinski *et al*., 2015), automatic continuous cough monitoring (Vandermeulen *et al*., 2016), infrared thermography (Schaefer *et al*., 2012), measurement of acute phase proteins - APPs (Wolfger *et al*., 2015).

APPs are synthesized during the innate immune response process, comprising homeostatic disorders caused by infection, tissue injury, trauma, surgery, neoplasms and immune disorders (Tóthová *et al*., 2015). Among the APPs used in herd health management, haptoglobin (Hp) is the most important for ruminants, as it can help in the diagnosis, monitoring and prognosis of diseases (Eckersall and Bell, 2010). Higher concentrations of Hp in the first week of calves' life were related to higher degrees of future diseases, as well as higher mortality rates. For each 1g/L increase in the Hp value, the chance of these animals needing to receive treatment for any disease, between 15 and 120 days of life increased 7.6 times (Murray *et al*., 2014). Windeyer *et al*. (2014) found that calves that received treatment for diarrhea and other diseases had lower rates of weight gain.

Thus, the detection of diseases by clinical evaluations and its possible association with blood Hp values led us to hypothesize whether this APPs could be used as a predictor of diseases in calves. Another important gap to be investigated is the association between the concentration of Hp with the development indexes of the calves in the first months of life since few studies have been developed. Therefore, the aim of this research was to evaluate the use of Hp as an indicator of health and performance in Holstein heifer calves reared in an intensive production system.

MATERIAL AND METHODS

This research was approved by the Ethics Committee on the Use of Animals - CEUA nº 5132061217, from the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ-USP), and conducted on a high production commercial farm, with 1750 cows in lactation and average daily milk production of 38.5L per cow, located in the municipality of Araras – SP (latitude 22º21’25’’S, longitude 47º23’03’’W), from May to December 2017.

Holstein heifer calves (n=166) were selected, between birth and 65 days of life, whose births occurred between June and October 2017. The pre-calving management of cows consisted of the vaccination schedule at the time of drying off (60 days pre-calving), and after 30 days against rotavirus, coronavirus, *E. coli* F5; transfer of pasture (dry cows) to the maternity paddocks 30 days before the estimated date of calving. Births were always supervised, calves received umbilical treatment and were transferred to individual shelters. The roof hygiene protocols, pre and post-dipping was performed, followed by colostrum milking in a cattle chute located in the maternity paddock.

Heifer calves were fed with a minimum volume of three liters of fresh colostrum containing at least 21% of total solids content, offered in the first two hours of life. When necessary, a second colostrum feed was offered in order to reach the total ingested volume equivalent to 10% of body weight in the first 18 hours of life. Frozen colostrum was offered in exceptional cases when the immunological quality of the maternal colostrum did not meet the goals pre-established by the farm. Umbilical cord antisepsis was performed by immersion in a 10% iodine solution, repeating the procedure at least once a day until mummification and detachment of the external umbilical cord. On the second day of life, calves were fed with seven to eight liters/day of pasteurized waste milk, divided into two feedings (morning and afternoon), in addition to water and concentrate ad libitum. The calves were kept in individual housing until the weaning.

The vaccination protocol of the calves consisted of the administration of a dose of the intranasal vaccine in the first week of life against Bovine Herpesvirus type 1 (BoHV-1), Bovine

Parainfluenza type 3 (PIV-3), and Bovine Respiratory Syncytial Virus (BRSV), with booster 30 days after the first vaccination. Heifer calves were evaluated at five intervals: D6-9 (six to nine days old); D10-13 (ten to 13 days old); D20-23 (20 to 23 days old), D35-38 (35 to 38 days old), and D65-68 (65 to 68 days old). Calf health was monitored for the presence or absence of diarrhea and BRD, following the procedures for classification by scores described in the Calf Health Scoring Criteria (Poulsen and Mcguirk, 2009), in addition to the physical examination of the umbilical region to detect inflammation (Dirksen *et al*., 1993).

For the evaluation of diarrhea, the stool consistency was classified into scores: normal (score zero); pasty/semi-formed (score 1); pasty with more water and feces adhered to the perineum and tail (score 2); liquid with fecal content adhered to the perineum and tail (score 3), with zero and 1 being the scores used for absence and 2 and 3 for the presence of diarrhea. For BRD detection, the parameters used (cough, nasal discharge, ocular discharge, ear positioning and temperature) were scored according to the intensity of the clinical manifestation (zero to three). At the end of the evaluation, the total score was determined from the sum of the points assigned to each parameter and the calves that presented a sum of the parameters >4.0 were considered positive.

The performance parameters (weight, height at withers and hind width) were evaluated in D10-13; D20-23, D35-38 and D65-68. The weight was obtained with the use of a tape suitable for ruminants, while the height at withers and width of the hind were measured using an anthropometric ruler. Blood samples were obtained by puncture of the jugular vein, in tubes without anticoagulant, for the measurement of haptoglobin (Hp), determined on its ability to bind with hemoglobin (Jones and Mould, 1984), using spectrophotometry and wavelength of 495nm. After the optical densities, the Hp concentrations (g/L) of the samples were determined using the least squares method, with function obtained in a polynomial regression of order 3, with the known values of the standard Hp, for each ELISA plate. The intra-test coefficient of variation was 7.25% and inter-test coefficient was 8.19%.

Statistical analysis was performed using the SPSS software, version 19. The distribution of the quantitative variable was presented using histograms and the Kolmogorv-Sminorv test. Numerical transformations were performed when necessary. The effect of time on the concentration of Hp was evaluated with the analysis of variance (one-way ANOVA) for repeated measures, with Bonferroni's post-hoc test. The evaluation of the effect of diseases (diarrhea, BRD, umbilical inflammation) on the concentration of Hp was performed at the peak of each disease. In the analysis of the association the effect of the diarrhea on the concentration of the Hp, the moment of the peak of the disease was selected and the animals with other diseases (BRD, omphalitis and others) were removed of the analysis. The association between BRD and the HP concentration was realized at the peak of the disease and the animals with diarrhea were removed of the analysis. The association between the omphalitis and Hp concentration was realized at the peak of this disease and the positive cases for other diseases were removed of the analysis.

The analysis of the effect of diseases on the concentration of Hp at the time of highest prevalence (peak) was performed using the T-student test for comparison between groups (healthy x sick), while analysis of variance (ANOVA) with Bonferroni post-hoc test allowed to evaluate the effect of the different scores of diarrhea (zero, 1, 2, 3), and the clinical parameters involved in the classification of BRD (cough, nasal discharge, eye discharge, ear positioning and temperature) on Hp concentration. The association of Hp with the performance parameters (weight, height at withers and width hind) was evaluated using a correlation model for repeated measures (rmcorr), in R-studio version 3.6.6.

RESULTS

The total rate of deaths during the experiment was 3% (5/166). The peak of diarrhea occurred in D10-13, with 59.4% (98/165) of positive cases, while in the other studied moments (D6-9; D23-25; D35-38; D65-68), the respective prevalence was: 6.6% (11/166); 9.1% (15/165); 6.7% (11/163), and 5.0% (8/161) (Figure 1A). The highest prevalence of BRD (25.8%, 42/163) was in D35-38 (Figure 1B). At other times (D6-9; D10-13; D23-25; D65-68), the prevalence wase

6.6% (11/166); 12.7% (21/165); 20.0% (33/165); 16.8% (27/161). Regarding umbilical inflammation (Figure 1C), the highest prevalence was observed in D6-9 (7.8%, 13/166), followed by D10-13 (4.8%, 8/165); D35-38 (3.1%, 5/163); D20-23 (3.0%, 5/165); and D65-68 (0%, 0/161).
Calves showed a significant increase in weight, height at withers and hind width over time (P<0.001). Weight ranged from 49.0±6.3kg in D10-13 to 97.1±10.8kg in D65-68 (Figure 1D). Height at withers varied from 71.4±3.4cm in the D10-13 to 85.5±3.4cm in D65-68 (Figure 1E). The width of the hind ranged from 20.9±1.9cm in the D10-13 to 26.1±1.4cm in the D65-68 (Figure 1F).

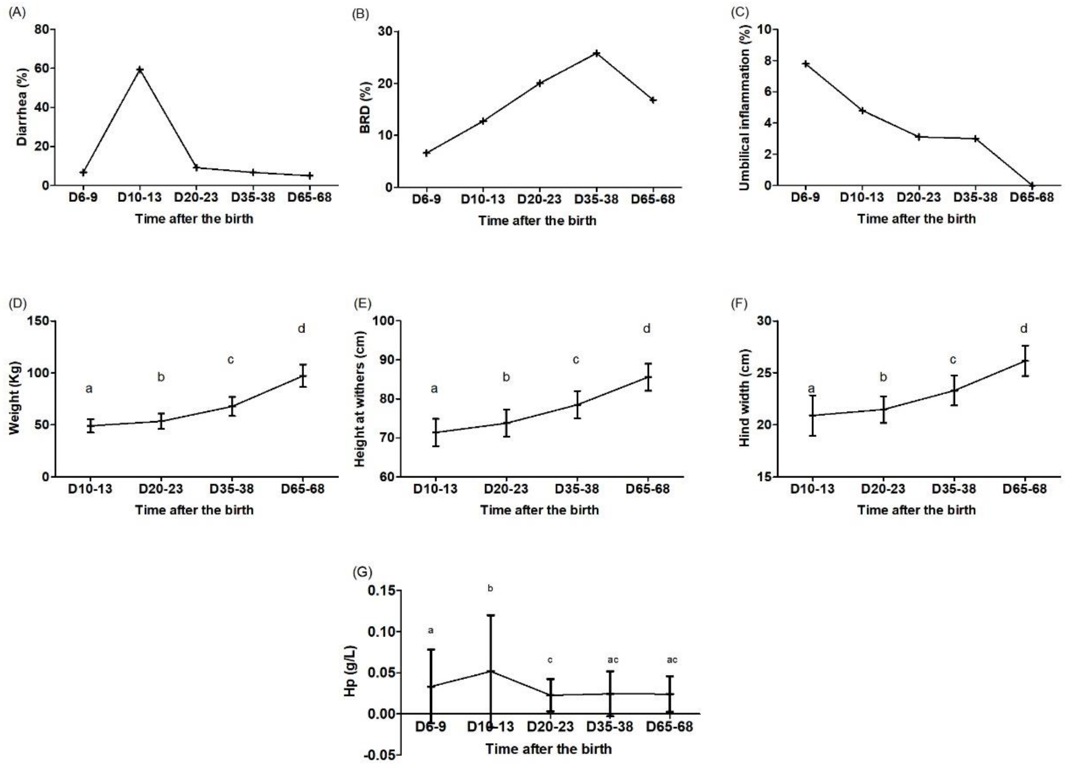

Figure 1. Profile of diarrhea (1A), BRD (1B), umbilical inflammation (1C), weight (1D), height at withers (1E), hind width (1F), and Hp (1G) in early life Holstein heifer calves. D=days; BRD=bovine respiratory disease; Hp=haptoglobin. Different letters indicate statistical differences obtained by analysis of variance (one-way ANOVA), for repeated measures, using the Bonferroni test.

Hp showed a significant variation over time (P<0.05), with an increase in D10-13 (0.05±0.06g/L), compared to D6-9 (0.03±0, 04g/L). In D23-25, the Hp concentration decreased significantly (0.02±0.02g/L) with subsequent stabilization in the subsequent moments (D35-38: 0.02±0.03g/L and D65-68: 0.02±0.02g/L) (Figure 1G). In the peak of the diarrhea (D10-13, n=53/100), the concentration of Hp (0.05±0.04g/L) was higher (P=0.02) than in the group of healthy calves (0.03±0.04g/L, 47/100) (Figure 2A). The different diarrhea scores influenced the Hp values (P=0.036): calves with score 3 (n=23/100) had a concentration of 0.06±0.05g/L, while calves with scores 2 (n=30/100), 1 (n=10/100) and zero (n=37/100) had concentrations of 0.04±0.04g/L; 0.06±0.07g/L and 0.03±0.02g/L, respectively. In the multiple comparisons it was possible to observe that the score 3 of diarrhea showed a significant difference (P=0.024) in relation to the score zero (Figure 2B).

The moment of highest prevalence of BRD was at D35-38, with a Hp value (0.03±0.05g/L) similar (P=0.244) to the group of healthy calves (0.02±0.01g/L). The individual evaluation of each score used for the classification of BRD (nasal and ocular discharge, head positioning, cough and temperature) was performed, and that there was an association between cough (P=0.009), and temperature (P=0.040) with the concentration of Hp (Figure 2C and 2D, respectively). Calves with score zero (absent) of cough had an Hp concentration of 0.02±0.01g/L; in score 1 (cough after stimulation) the concentration was 0.03±0.04g/L and in score 2 (spontaneous cough), 0.03±0.02g/L.

There was a difference between the values obtained for the score zero and 1 (P=0.016). Regarding temperature, calves with score zero (37.7-38.2°C), and 1 (38.3-38.8°C) had Hp value of 0.02±0.01g/L, in the score 2 (38.9-39.3°C) the value was 0.02±0.02g/L and in score 3 (≥39.4°C) the value was 0.05±0.06g/L. The analysis of multiple comparisons allowed to observe that the concentration of Hp for calves with temperature score 3 was higher (P=0.049) when compared to the score zero (Figure 2D). Hp values were higher in animals with umbilical inflammation at D6-9 (0.05±0.05g/L), whereas healthy animals showed a concentration of 0.03±0.02g/L, making it possible to identify a statistical trend (P=0.057, Figure 2E).

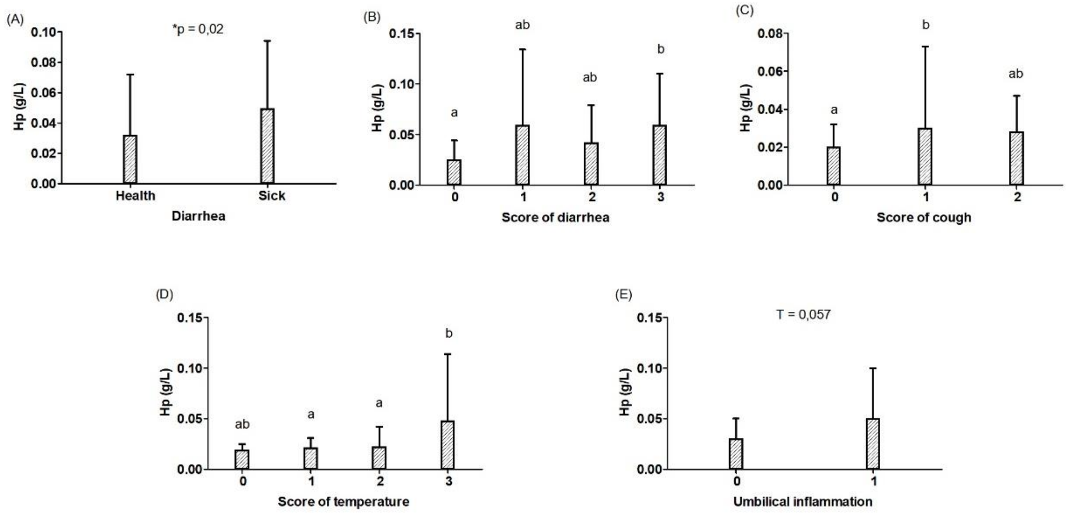

Figure 2. Haptoglobin (Hp) concentration in calves with and without diarrhea (A), and according to different scores of diarrhea (B), different degrees of cough (C), different degrees of body temperature (D), and umbilical inflammation (E) at the peak of the disease (peak of diarrhea=D10-13 and peak of BRD=D35-38). Diarrhea scores: 0=without diarrhea; 1=pasty / semi-formed stools; 2=pasty stools with more water and feces adhered to the perineum and tail and 3=liquid with fecal content adhered to the perineum and tail. Cough scores: 0=absent; 1=present when stimulated; 2=spontaneous cough. Temperature scores: 0=37.7-38.2ºC; 1=38.3-38.8ºC; 2=38.9-39.3ºC; 3=≥39.4ºC. Score of umbilical inflammation: 0=without alterations; 1=with inflammatory alteration. Different letters indicate significant differences by the Bonferroni test. *p=p-value<0.05 and T=statistical trend (p-value between 0.05 and 0.1) obtained by test T for independent samples.

The correlation analysis for repeated measures (rmcorr) revealed a significant and negative association between the concentration of Hp with weight, height at withers and hind width (P=0.001). The correlation coefficient between Hp and weight was -0.187 (95% CI=-0.272 / -0.098). The relationship between Hp and height at the withers was -0.178 (95% CI=-0.265 / -0.089). The association between Hp and the hind of the width was -0.172 (95% CI=-0.259 / -0.084).

DISCUSSION

This work presented the profile of diarrhea, BRD, umbilical inflammation and Hp in calves up to 65 days of age, in addition to the association of Hp with these diseases and the performance of the calves. The mortality rate of this research (3%) was similar to Windeyer *et al*. (2014), who evaluated calves up to three months of age (3.5%). The highest prevalence of diarrhea (59.4%) was found between 10 and 13 days of age, this value being lower than the prevalence observed by

Novo *et al*. (2017), who evaluated Holstein calves aged between seven and 14 days old (68.9%). The study by Novo *et al*. (2017) was carried out on the same farm as the present study, and the management changes implemented after the determination of these prevalence, mainly with the inclusion of halofuginone-based substances for the control of diarrhea by *Cryptosporidium* sp. and the inclusion of chlorine dioxide in the disinfection of the calf housing impacted on the reduction of diarrhea rates observed in the present study. In the work of Seppä-Lassila *et al*. (2015) in Finland, the prevalence of diarrhea for calves aged 15 to 60 days of life was 32.6%.

The prevalence of BRD was higher in D35-38, with 25.8%, a value higher than that obtained in Canada involving 19 dairy farms, in calves that were followed from the first to the 110th days of life. In this study, the interval between 37 to 90 days of life was the period with the highest occurrence - 9.5% (Windeyer *et al*., 2014). Lago *et al*. (2006) found an overall prevalence of 14% in 13 studied farms, with the highest prevalence observed in the seventh week of life (27.0%), being similar to the value obtained in our study.

Regarding the prevalence of umbilical inflammation, it is known that the general conditions of management and health of heifer calves interfere in the prevalence, which can vary from 1.3% (Svensson *et al*., 2007) to 30.0% (Novo *et al*., 2017). Differences in geographical location; management adopted among farms; environmental risk factors (climate, humidity, dust particles, stocking, ventilation); and epidemiological factors (different actions from etiological agents, mode of transmission, periods of infection) may have promoted a divergence in relation to the rates observed in the studies cited. Factors linked to the host (stress, low body weight, diversity between the age groups studied, failure to ingest colostrum) are also important factors that can interfere in these values (Snowder *et al*., 2006).

Windeyer *et al*. (2014) found that approximately 20% of the cases of BRD could have been avoided if the colostrum supply had been adequate. It is known that animals become more susceptible in the period considered as an immunological window, which involves the transition from passive to active immunity (Chase *et al*. 2008). In this period, antibodies from maternal colostrum reduce significantly in the circulation of calves due to their half-life, that varies from 21.2 to 35.9 days after birth (Fulton *et al*., 2004), a period that includes the peak of respiratory disease verified in this research.

In addition to the immunological window, the effectiveness of maternal antibody transfer depends on factors such as the ingested and absorbed concentration of colostrum, the time of supply after birth and quality of colostrum (Chase *et al*., 2008). In the present study, the heifer calves received fresh and frozen colostrum, the latter being offered just when the immunological quality of the maternal colostrum did not meet the farm goals. Novo *et al*. (2017) found clinical signs of more intense diarrhea and umbilical infections in calves that received frozen colostrum, that is, free of maternal cells, responsible for improving the immune response of newborns, with greater bactericidal activity in neutrophils (Reber *et al*., 2008). Despite the vaccination management procedures, calving assistance and navel antisepsis adopted on the farm studied in this research, it appears that they were not sufficient to prevent the prevalence of diseases evaluated here, with the provision of frozen colostrum being a favorable possibility for such fees.

The etiologic agent involved can also interfere in the prevalence. The main primary agents of BRD that may be associated with the peak of the disease are the Bovine Herpesvirus type 1, Bovine Viral Diarrhea virus, Parainfluenza-3 virus, Bovine Respiratory Syncytial virus, and Coronavirus. Secondary infections are caused by the bacteria *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* (Woolums *et al*., 2009). Considering the Hp profile in relation to the diseases evaluated, it was found that the highest value obtained for diarrhea was in D10-13 (peak of the disease), with this value being different from that obtained for Hp in the group of healthy animals.

Hajimohammadi *et al*. (2013) found higher Hp values in Holstein calves that presented severe diarrhea (0.47g/L), when compared to moderate (0.26g/L) or without clinical signs (0.24g/L). This result supports what was verified in the present study, where calves that presented score 3 of diarrhea (liquid feces with fecal content adhered to the perineum and tail) had higher Hp values (0.06g/L), when compared with heifer calves that showed no changes (0.03g/L) in the consistency of feces (score zero). Despite the Hp profile reflecting that its variation occurred according to the intensity of the infection (Horadagoda *et al*., 1999), the values obtained between the two surveys were quite different, which can be justified by the differences in the pathogenicity caused by each agent causing the diarrhea (Pourjafar *et al*., 2011), since in both studies they were not determined, evaluating only the clinical score of stool consistency. The main etiological agents of diarrhea in dairy calves are *E. coli* K99 (F5), *Salmonella* spp, rotavirus, coronavirus and *Cryptosporidium parvum*, promoting inflammation of the intestinal epithelium and consequent secretory or malabsorptive diarrhea (Foster and Smith, 2009).

Another factor that can lead to the difference between the values is the type of test used to measure Hp, in the Hajimohammadi *et al*. (2013) study, a commercial kit (Tridelta Development) was used, while in our research spectrophotometry was used. Regarding BRD, there was no difference in Hp values between sick and healthy animals. On the other hand, it was found that animals that presented cough after stimulation and body temperature >39.4°C had higher values of Hp, when compared to animals without cough and body temperatures between 37.7-38.2°C. Murray *et al*. (2014) found a high association between Hp with the development of fever and a higher intensity of the scores used to detect disease. Svensson *et al*. (2007) found in their study a low ability of Hp to predict BRD in calves, however, the sensitivity and specificity of the test increased significantly when body temperature was combined with Hp. The variation in sensitivity and specificity between the tests that measure Hp, associated with factors that can change their values even in the absence of infection, reinforce the importance of the association of several parameters when the objective is to evaluate a certain disease. Temperature and Hp are not sufficient to establish a specific diagnosis, but they can provide objective information about the intensity and behavior of individual injuries.

Many studies have shown that Hp may have an important potential in helping to detect diseases (Murray *et al*., 2014; Seppä-Lassila *et al*., 2015; Tóthová *et al*., 2015). However, the need for studies evaluating the accuracy of Hp in face of cases of BRD was reported in a systematic review (Abdallah *et al*., 2016). The results of the different articles showed great variations in relation to sensitivity and specificity, due to the different methodologies employed. The present study showed differentials with the use of selection criteria for sick and healthy animals, in addition to an accurate definition of the methodology for detecting Hp.

Few studies have evaluated the relationship between the concentration of APPs and the average daily weight gain, and as far as it was possible to verify, no work until then had evaluated the association of Hp with performance indexes. Murray *et al*. (2014), and Pardon *et al*. (2015) found no association between these parameters, however, Niine *et al*. (2018), like the present study, found a negative association between Hp and average daily weight gain in calves treated for infections by *Cryptosporidium* spp. The negative relationship verified in this research between the Hp profile with the calves' performance (body weight, height at withers and hind width) reinforces the impact of diseases on the animal's organism, reflecting on its development. Therefore, for these parameters, Hp was shown to be an important predictive potential, but considering the scarcity of studies, future studies should establish cutoff points and assess the relationship of Hp concentration at the beginning of life with the development of calves for better clarification of the associations observed in this study.

The knowledge of diseases profile in intensive production systems is essential for the adoption of strategies to reduce their occurrence, and the use of Hp as a biomarker - considering the results obtained in this study - can be better used in diarrhea, when compared to BRD or umbilical inflammations. The negative relationship of Hp with the development of calves opens important perspectives on the use of this APPs not only as a biomarker of diseases, but also as a developmental biomarker. The prediction of diseases, development and mortality are fundamental for the adoption of strategies to minimize losses in milk production.

CONCLUSIONS

Calves with diarrhea showed higher concentrations of Hp. There was a negative association of Hp with the performance indexes of the calves. This study contributed to a better understanding of the association of diseases and performance with Hp in Holstein heifer calves and highlights perspectives for possible applications of its use as a biomarker of diarrhea and performance in an intensive production system.

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