UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

ANALYSIS OF BUFFALO COLOSTRUM AND BLOD SERUM FROM BUBALINE CALVES WITH ENFASIS ON PASSIVE IMMUNITY TRANSFER

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Médico Veterinário

2019

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em: Medicina Veterinária, área: Clínica Médica Veterinária.

2019

S729a

Souza, Damazio Campos de
Analysis of buffalo colostrum and blood serum from bubaline calves to assess passive immunity transfer and growth performance / Damazio Campos de Souza. -- Jaboticabal, 2019
74 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: José Jurandir Fagliari
Coorientador: Rinaldo Batista Viana

1. Medicina veterinária. 2. Imunologia veterinária. 3. Imunoglobulina G. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: ANALYSIS OF BUFFALO COLOSTRUM AND BLOD SERUM FROM BUBALINE CALVES WITH ENFASIS ON PASSIVE IMMUNITY TRANSFER

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Jaboticabal, 12 de novembro de 2019

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DAMAZIO CAMPOS DE SOUZA, Born March 7, 1990, in Belém, Pará, Brazil. Son of Solange Maria Cavalcante Campos and José Aparecido de Souza. Graduated in Veterinary Medicine from the Federal Rural University of Amazonia (UFRA) in March 2014. During graduation, he was member of the Programa de Educação Tutorial (PET) of Veterinary Medicine from September 2009 until March 2014 with scholarship of Secretaria de Ensino Superior – Ministério da Educação (SESu\MEC). He began a Master's on Veterinary Medicine (Clinical Veterinary Medicine) at São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, beginning in March 2014 and ending February 2016, under the guidance of Prof. Dr. José Jurandir Fagliari, with scholarship of the São Paulo Research Foundation (FAPESP) Process: 2014/09246-5. He started his PhD on Veterinary Medicine (Clinical Veterinary Medicine) at the same university in March 2016 with the same guidance and with scholarship of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)-Finance Code 001.

Dedico à memória da minha amada avó
Juraci Cavalcante Campos.

AGRADECIMENTOS

A Deus e à minha Nossa Senhora, pela força, acalento, sentido e motivação, pois sem eles não teria a chance de estar aqui podendo construir minha existência.

À memória de meu avô Prof. Nicolino de Castro Campos, homem que me ensinou que o maior tesouro de um homem é o seu conhecimento.

À memória de meu pai, José Aparecido de Souza, um trabalhador brasileiro, que lutou uma vida, não obstante o adversário. Se hoje eu não tenho medo é graças ao senhor Pai.

À Solange Maria Cavalcante Campos, minha mãe, que nunca mediu esforços para minha evolução pessoal e profissional. Que sentou do meu lado me estimulando para aprender a ler e escrever. Médica Neonatologista, de quem busquei inspiração nos cuidados e carinhos com os espíritos recém-chegados, sempre trabalhando com amor mesmo diante das adversidades. Se hoje sou o cidadão que sou, é graças ao seu exemplo.

À minha irmã, Camila Campos de Souza, amiga, companheira e sempre paciente, muito obrigado pelo amor ao longo dos anos.

Ao Prof. José Jurandir Fagliari, homem, pai, veterinário, professor, difusor de conhecimento. Que me permitiu a oportunidade do crescimento por conta própria, respeitando minhas limitações; sempre calmo e sereno.

Ao Prof. Rinaldo Batista Viana, dileto orientador, que me trouxe a oportunidade de desenvolvimento na Buiatria desde o início de minha formação.

A Dra. Daniela Gomes da Silva, sempre atuante no laboratório. Sou muito grato por todos os ensinamentos e pela presença certa em todas as fases de desenvolvimento do projeto.

Às bolsistas de iniciação científica Lana Cristina Coelho Fonseca e Letícia de Castro Fiori pelo trabalho em conjunto no laboratório e tabulação dos resultados obtidos.

Ao Prof. Bruno Moura Monteiro, amigo que carrego comigo. Que me recebeu pela primeira vez na cidade de São Paulo. Conselheiro durante todo o percurso, quem trouxe calma e aprumo nos momentos mais necessários, responsável pelo estreitamento dos laços na propriedade. Grande profissional em quem busquei inspiração na escolha do percurso acadêmico.

Ao Dr. Otávio Bernardes, exemplo de homem, profissional, empreendedor. Um daqueles grandes gênios multivalentes, destemido, com capacidades nas mais diversas

áreas do conhecimento. Sou grato por ter aberto as porteiras da fazenda, as portas de sua casa, mas ainda mais pela confiança no meu trabalho, apoio na adversidade e principalmente pelos ensinamentos e pelo exemplo de que a expansão do conhecimento jamais deve ser limitada pela área de atuação profissional.

À memória do Dr. Osmar Bernardes, pela ajuda inestimável em todos os momentos de necessidade, pela confiança no trabalho realizado e pelas palavras de apoio.

À Eneida Bernardes, pela recepção, amabilidade e atenção dispendidas ao longo do período de estudo, que foram essenciais para que o trabalho se concretizasse.

Aos técnicos e amigos do Laboratório de Pesquisa do Departamento de Clínica e Cirurgia Veterinária da FCAV - UNESP, Renata Lemos Nagib Jorge, Cláudia Aparecida da Silva Nogueira e Paulo César da Silva, pela ajuda, paciência, e amizades construídas.

Aos veterinários Gustavo Lara e Marina Migliano, pela ajuda, pelas informações do manejo e oportunidades de total integração com a fazenda.

Aos companheiros de trabalho na fazenda, José Augusto, Rodrigo, Izael, Marco, Jonathan e Antônio, pessoas que dedicam a vida à produção animal, pela amizade, companheirismo e por me tratarem como irmão.

Às crianças da fazenda, Richard, Lucas, Daniel, Kauanne, Mateus e Isaac. Pelo amor e carinho puros e verdadeiros, muito obrigado por me fazerem sentir em casa, o tio ama vocês e nunca vai esquecer do que vocês me ensinaram.

Aos membros da Comissão Examinadora de Qualificação, Profa. Dra. Lindsay Unno Gimenes e ao Prof. Dr. Estevam Lux Hoppe, pelas correções e sugestões pertinentes que contribuíram sobremaneira com este trabalho.

Aos meus estagiários, Feijão, Pitica e Rajada, companheiros durante todo o período do experimento, pela companhia silenciosa e fiel que me seguia por toda fazenda desde o nascer ao pôr-do-sol.

Aos búfalos utilizados no experimento, mães e bezerros, foi uma experiência única e reveladora acompanhar o crescimento desses animais diariamente, mesmo após centenas de quilos ganhos ainda eram os mesmos bezerros que eu carregava nas costas, que corriam na porteira do piquete ao me verem chegar.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Sou grato a todos.

SUMMARY

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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CERTIFICADO

Certificamos que o Projeto intitulado **"Análise do colostro de búfalas e do soro sanguíneo de bezerros bubalinos para avaliação da transferência de imunidade passiva e da cinética das proteínas de fase aguda"**, protocolo nº 17.366/16, sob a responsabilidade do Prof. Dr. José Jurandir Fagliari, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 14 de dezembro de 2016.

Vigência do Projeto	10/01/2017 a 10/01/2018
Espécie / Linhagem	*Bubalus bubalis* (búfalo-do-rio) / Murrah
Nº de animais	200
Peso / Idade	30 a 650 Kg
Sexo	Macho e fêmea
Origem	Fazenda Paineiras do Ingaí – Alambari/SP

Jaboticabal, 14 de dezembro de 2016.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
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ANÁLISE DO COLOSTRO DE BÚFALAS E DO SORO SANGUÍNEO DE BEZERROS BUBALINOS PARA AVALIAÇÃO DA TRANSFERÊNCIA DA IMUNIDADE PASSIVA

RESUMO - O objetivo deste estudo foi avaliar a transferência de imunidade passiva em bezerros bubalinos saudáveis e avaliar a influência das concentrações séricas de imunoglobulina G (IgG) às 24 horas no desempenho produtivo nos primeiros 90 dias de idade dos bezerros. Amostras de colostro de novilhas (sem parto prévio), primíparas e pluríparas; e amostras de sangue de seus respectivos bezerros foram coletadas no nascimento, antes da ingestão de colostro, e 24h, 48h e 72h após o nascimento para determinação das atividades séricas de gamamaglutamiltransferase (GGT) e fosfatase alcalina (ALP); e concentrações séricas de proteína total (PT), imunoglobulinas A (IgA) e G (IgG) e lactoferrina. As concentrações de imunoglobulinas de amostras de colostro de búfalas e amostras de sangue de bezerros foram determinadas pela técnica de eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio (SDS-PAGE). O peso foi medido a cada semana após o nascimento até os três meses de idade. Os resultados da transferência de imunidade passiva foram analisados como medidas repetidas no tempo e o peso e o ganho de peso diário (GPD) foram avaliados por regressão linear simples. As diferenças foram consideradas significativas quando $P \leq 0,05$. Os búfalos nasceram hipogamaglobulinêmicos (4,23±0,33 mg/mL), e às 24 horas as concentrações séricas de IgG foram de 34,5±1,48 mg/mL, a transferência de imunidade passiva foi considerada bem-sucedida. As concentrações séricas de PT são recomendadas como parâmetros indiretos confiáveis para avaliar a transferência de imunidade passiva em búfalos. O peso médio ao nascimento foi de 38,0±5,54 Kg, e o peso e o GPD aos 30, 60 e 90 dias de idade foram respectivamente: 49,2±6,30 Kg; 61,7±8,14 Kg; 75,9±10,3 Kg e 0,363±0,139 Kg/d; 0,391±0,101 Kg/d; 0,421±0,091 Kg/d. Não foi detectada relação significativa das concentrações séricas de IgG às 24h com o peso ou GPD durante o período do estudo. Os resultados produtivos de bezerros bubalinos nos primeiros 90 dias de idade são mais influenciados pela

nutrição da mãe, características genéticas herdáveis e bom manejo sanitário dos bezerros.

Palavras-chave: bezerro, colostro, Murrah, neonato, recém-nascido

ANALYSIS OF BUFFALO COLOSTRUM AND BLOOD SERUM FROM BUBALINE CALVES TO ASSESS PASSIVE IMMUNITY TRANSFER AND GROWTH PERFORMANCE

ABSTRACT – The aim of this study was to evaluate the passive immunity transfer on healthy buffalo calves and evaluate the influence of serum concentrations of immunoglobulin G (IgG) at 24hs on productive performance on the first 90 days of age of Murrah buffalo calves. Colostrum samples from heifers (without previous calving), primiparous and pluriparous dams; and blood samples from their respective calves, were taken at birth, before colostrum ingestion, and at 24h, 48h, and 72h after birth for determination of serum activities of gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP); and serum concentrations of total protein (TP), immunoglobulins A (IgA) and G (IgG) and lactoferrin. The concentrations of immunoglobulins of colostrum samples from buffaloes and blood samples from calves were determined by sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE) technique. Weight was measured following each week after birth until three months of age. The passive immunity transfer results were analyzed as repeated measures and weight and average daily gain (ADG) were evaluated by simple linear regression. Differences were considered significant when $P \leq 0.05$. As buffalo calves were born hypogammaglobulinemic (4.23±0.33 mg/mL), and at 24h serum concentrations of IgG were of 34.5±1.48 mg/mL, passive immunity transfer was successful. Serum concentrations of TP at 24h is recommended as reliable indirect parameter to evaluate passive immunity transfer on buffalo calves. An average weight at birth was of 38.0±5.54 Kg, and weight and ADG with 30, 60 and 90 days of age were respectively as follows: 49.2±6.30 Kg; 61.7±8.14 Kg; 75.9±10.3 Kg and 0.363±0.139 Kg/d; 0.391±0.101 Kg/d; 0.421±0.091 Kg/d. No significant relation between serum concentrations of IgG at 24h and weight or ADG was detected. The production outcomes of buffalo calves on the first 90 days of age are more influenced by the dam's nutrition, genetic heritable characteristics and good health management of the calf.

Keywords: calf, colostrum, Murrah, neonate, newborn

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CHAPTER 3 – Relationship between productive performance and serum concentrations of immunoglobulin G of Murrah calves allowed to nurse the dam

CHAPTER 1 – General considerations

1. Introduction

The buffalo (*Bubalus bubalis*) is a species with deep roots within Pacific and Atlantic countries, being essential part of the livelihood on developing countries, and achieving greater importance under recent climate changes, where producers are transitioning from crop to livestock production (Escarcha et al., 2018).

The estimated buffalo world stock is of 199 million animals (FAO, 2019). With Brazil as the biggest producer on America with 1,381,345 heads and a milk production of 100 million L/year with estimated market value of US$300 million (EMBRAPA, 2018).

Neonatal period is critical to buffalos being the phase were most loss occur, with high mortality rates ranging from 17% to 36% (Pardhan and Panda, 1994; Shivahre et al., 2014). The most efficient way to ensure low mortality levels of dairy calves is to make sure that passive immunity transfer (PIT) is done within the first hours after birth, meaning that calves have at least 20–25 mg/mL of serum concentrations of immunoglobulin G (IgG) at 24h (Chigerwe et al., 2015).

As ruminants, buffalo calves are born agammaglobulinemic or hypogammaglobulinemic with little to none transplacental passage of immunoglobulins (Ig) (Singh et al., 1993; Souza et al., 2019). The way that maternal antibodies reach the fetus is determined by placental structure. In ruminants with sinepteliocorial placentation, where the chorionic epithelium is in direct contact with uterine tissues, there is an impediment to the passage of immunoglobulins (Igs) (Tizard, 2009).

Benchmarking individual PIT values on each farm has been proved to enhance production outcomes as better management practices can be adopted to ensure PIT (Beam et al., 2009; Atkinson et al., 2017). Also, high concentration of Igs had been proven to result in higher growth performance values (Mastellone et al., 2011; De Paula et al., 2019)

The measurement of IgG on both dam’s colostrum and calf’s blood serve as a tool to evaluate colostrum management practices. Taking this in account,

the present research aimed to evaluate the passive immunity transfer of buffalo calves, by evaluating colostrum from dams and the blood serum of calves in the first three days of age.

In addition, the study aimed to access the association of IgG at 24h and growth performance in buffalo calves from birth up to 90 days of age.

Also, serum biochemical parameters of buffalo calves where studied from birth until three months of age to determine their biochemical profile.

2. Literature Review

Colostrum is composed by: fat, protein, vitamins, minerals, lactoferrin, immune cells, cytokines and immunoglobulins (Ig). Colostrogenesis is the passage of Igs from the bloodstream to the mother's mammary secretion. In ruminant females, this transfer begins several weeks and peaks from 1–3 days before delivery. The end of Igs passage is probably linked to the rise in prolactin serum concentrations (Barrington and Parish, 2001; Barrington et al., 2002).

Immunoglobulin G (IgG) represents 86% of the total Igs on buffalo colostrum, completed by immunoglobulin A (IgA) and immunoglobulin M (IgM) 8% and 6% respectively (Dang et al., 2009). The predominance of IgG is due to active and selective FcRn receptors in the epithelium of the mammary gland; those same receptors are present in the intestinal epithelial cells of the calf and carry IgG through endocytosis to the blood circulation (Chaudhary et al., 2018). However, the transfer of other classes of immunoglobulins is probably not selective reaching lower levels in colostrum. IgG is capable of agglutination, opsonization, inhibition of the adhesion of pathogens on the endothelial surface of intestine, complement fixation and neutralization of toxins; IgM has the same properties but with enhanced complement fixation; while IgA acts to prevent infections through agglutination of microorganisms, binding to the intestinal wall receptors (Tizard, 2009).

Although most of the protection to the newborn are given by colostrum IgG, there are non-specific milk factors that ensure the optimal health of the calf like lactoferrin, lactoperoxidase, lysozyme and also xanthine dehydrogenase (XDH),

lipoprotein lipase (LPL) and pancreatic ribonuclease (RNASE1) responsible for maturation of the gastrointestinal tract (Zhang et al., 2015).

Absorption of immunoglobulins is not selective for specific types of immunoglobulins; in newborn calves the proteolytic activities in the digestive tract are still very low, being further reduced by the activity of trypsin inhibitors in colostrum. Thus, the proteins are not degraded and reach intact the small intestine, where they bind to receptors present in the intestinal epithelial cells (FcRn) and through endocytosis reach the blood circulation (Tizard, 2009).

Passive immunity transfer (PIT) is affected by colostrum quality and absorption by the calf. The timing of colostrum ingestion, the method and volume of colostrum administration, the presence of the dam, and respiratory acidosis of the newly born are linked to calf absorption (Weaver et al., 2000). Whereas, breed, nutrition, calving season, maternal vaccination, dry-off period, and calving intervals are determinants of colostrum quality (Burton et al., 1984; Pritchett et al., 1991; Quigley and Drewry, 1998; Morin et al., 2001).

Immunoglobulin transfer is optimal in the first 4 hours postpartum and begins to decline rapidly after 12 hours postpartum. The intestinal permeability to immunoglobulins decreases, probably due to the replacement of intestinal epithelial cells expressing FcRn by those that do not express these receptors. Calves fed earlier will have significantly higher serum IgG concentrations than those fed later when similar concentrations and volumes of colostrum are fed (Weaver et al., 2000; Tizard, 2009).

The IgG serum concentration in buffalo calves increases rapidly after feeding colostrum, peaks between 1 and 3 days of age, and then decreases. Samples should be collected between 2 and 7 days old to provide the most accurate indication of passive transfer (Elizondo-Salazar and Heinrichs, 2009; Souza et al., 2019)

The measurement of IgG serves as a tool to evaluate colostrum management. A failure of passive immunity transfer (FPIT) prevalence of <10% is considered as a rational and achievable goal to dairy farms when using a cut-off value of 10 g IgG/L (Chigerwe et al., 2009). The odds of FPIT are higher when there is no on-farm routine screening (Beam et al., 2009). Therefore, IgG

concentration of 48-hours-old calves should be estimated regularly to test the compliance of the colostrum management (Meganck et al., 2014).

The most common methods to assess PIT status in domestic animals, measuring direct IgG concentrations are the single radial immunodiffusion (SRID), used as gold standard, and the enzyme-linked immunosorbent assay (ELISA), even if those methods are not feasibly correlated (Dunn et al., 2018). While SDS-PAGE, refractometry, sodium sulfite or zinc sulfate turbidity test, estimate serum IgG concentration based on overall protein concentration (Gapper et al., 2007). Moreover, there are new on-farm methods like the split trehalase immunoglobulin G assay (STIGA) being validated (Drikic et al., 2018)

The protein fractionation using polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) allows the identification of up to 20 to 30 protein bands with molecular weights ranging from 24 to 340 kDa. SDS-PAGE is easy to perform with low cost requires a microquantity of sample and allows the visualization of extremely low protein concentrations. This allows the evaluation of several acute phase proteins of clinical interest and the identification and quantification, of Igs and had already been proven as a reliable method to estimate IgG concentrations (Fagliari et al., 2006; Eckersall, 2008).

In all species the neonatal period represents a critical moment where all organs must adapt to extrauterine life, a difficult transition from protection intrauterine for the adaptation to the life in the environment (Piccione et al., 2009).

The knowledge of the normal values of serum biochemical parameters is important in assessment of organ and tissue damage in different conditions and well-being assessment because it allows to monitor the metabolic condition of the animal tissues, the disorders in organ functions and body adaptation to nutritional and physiological changes (Kaneko et al., 2008).

The total protein (TP) is an indirect parameter, but with reliable correlation, of the amount of IgG (Borges et al., 2001). Serum concentration of TP values above 4.2 g/dL have high sensitivity and specificity in the detection FPIT on bovines. The assumption is based on the fact that the albumin contents of the newborn are little variable and that the differences in protein concentrations are

almost exclusively due to the absorption of immunoglobulins after colostrum ingestion (Jain, 1993; Feitosa et al., 2001; Radostits et al., 2007).

Measurement of gamma-glutamyltransferase (GGT) serum activity may also be used for the indirect assessment of PIT in calves. GGT activity is elevated in cow colostrum and its serum activity in calves fed with colostrum is 60 to 160 times greater than that observed in cows, and there is a significant correlation with serum IgG concentration (Fagliari et al., 1996; Radostits et al., 2007). In buffalo calves, GGT showed a correlation with TP and globulin values on the second and third day after birth, and can be used as a method to evaluate colostrum intake (Lombardi et al., 1996).

Aspartate aminotransferase (AST) is an enzyme that has high activity in skeletal muscles, heart muscle, and liver. It is present both in the cytoplasm of the cells and in the mitochondria. AST measurement, together with creatine kinase (CK), is used in the diagnosis of muscle tissue damage (Kaneko et al 2008). The increase in the serum activity of hepatic AST allows the detection of pathological processes associated with necrosis, as well as damage to the hepatic tissue (Ramaiah, 2007). In neonatal buffalo calves the dynamics of this protein is unknown.

Lactoferrin is the only plasma protein that transports iron; in addition, it has other functions like antiviral and antibacterial activities, besides acting like factor of growth. This protein is also capable of binding to surface receptors, mediating the entry of iron into cells (Kaplan et al., 1991).

The use of alkaline phosphatase (ALP) as a diagnostic marker is mainly related to liver function; however, it is also important in the evaluation of bone tissue. In the serum of young animals with high growth rates predominates the isoenzyme of bone origin (Kaneko et al, 2008). In calves on the first week of age the ALP of colostral origin predominates; however it has a low correlation with the amount of immunoglobulins acquired from colostrum (Rocha et al., 2012).

Acute phase proteins (APPs) are named this way because their plasma concentration changes rapidly in inflammatory diseases, after surgical trauma and in stress conditions (Jain, 1993; Murata et al., 2004; Wael and Sabry, 2014). These proteins are considered potential indicators of disease or as a marker of

animal welfare, either individually or in the herd, and can be an important tool in the diagnosis of animal diseases (Murata et al., 2004; Youssef et al., 2015). APPs whose serum concentrations decrease in response to inflammation are named negative APPs and include albumin and transferrin. APPs whose concentrations increase against the inflammatory stimulus are known as positive APPs and include C-reactive protein, α1-acid glycoprotein, α1-antitrypsin, α1-antichymotrypsin, serum amyloid A, ceruloplasmin, haptoglobin, α2 - macroglobin, fibrinogen, and components of complement (Eckersall, 2008).

Haptoglobin binds to free hemoglobin, inhibiting its oxidative activity (Yang et al., 2003). In addition to reducing the availability of the heme fraction of hemoglobin used for bacterial growth (Murata et al., 2004), this protein has high sensitivity for detecting inflammatory and/or infectious diseases in ruminants. In healthy animals their serum concentration is very low or undetectable (Eckersall, 2008); raising rapidly, within 24-48 hours after tissue damage, and may even detect animals with unapparent or subclinical infection (González et al., 2007). In calves, the haptoglobin concentration can be used to characterize the severity of diarrhea, as well as serve as a prognostic tool and assist in the decision and monitoring of the treatment (Hajimohammadi et al., 2013).

Ceruloplasmin has antioxidant and cytoprotective capacity, acting as an anti-inflammatory agent, reducing the adhesion of neutrophils to the vascular endothelium and acting in the destruction of the peroxidase; is used as an indicator of infections in cattle (Segelmark et al., 1997).

The α1-acid glycoprotein binds to several endogenous metabolites and acts on the innate immunity against infections, modulating the immune reaction by means of neutrophil activation and the inhibition of phagocytosis and platelet aggregation. This protein has a moderate to low response in response to tissue damage in cattle, increasing more slowly, with elevation usually associated with chronic conditions (Eckersall, 2008).

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the ALP of colostral origin predominates; however it has a low correlation with the amount of immunoglobulins acquired from colostrum (Rocha et al., 2012).

The evaluation of serum iron content is important because this mineral participates in several metabolic processes, such as hematopoiesis, hemoglobin synthesis, activation of the cellular immune response and pathogen-host interaction. It is directly related to the weight gain and growth of the animal, since it regulates the insulin-like growth factor type 1 (IGF-1) (Kaneko et al 2008; Prodanovic et al., 2014). While bovine calves require supplementation of iron (Atyabi et al., 2006), buffalo calves not, since there is a higher concentration of iron in buffalo milk when compared to cow's milk: 61 ppm versus 37 ppm (Verruma and Salgado, 1994).

Nevertheless, concerning the importance of the buffalo species to Brazilian dairy production there is little knowledge on the passive immunity transfer (PIT) of buffaloes. The second chapter of this thesis describes the PIT on Murrah buffalo calves, the third chapter evaluates the association between serum concentrations of IgG at 24h and growth performance parameters from birth until 90 days of age, and the fourth chapter describes the serum biochemical profile of buffalo calves.

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CHAPTER 2 – Passive immunity transfer in water buffaloes (*Bubalus bubalis*)[1]

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ABSTRACT - This study aimed to evaluate passive immunity transfer in healthy buffalo calves. Colostrum samples from heifers (without previous calving), primiparous and pluriparous dams and blood samples from their offspring were obtained at birth, before colostrum intake, and at 24, 48, and 72 h after birth for determination of serum activities of gammaglutamyltransferase and alkaline phosphatase and serum concentrations of total protein (TP), immunoglobulin A (IgA) and IgG, and lactoferrin. The results were analyzed as repeated measures, and differences were considered to be statistically significant at $P \leq 0.05$. Considering that the buffalo calves were born hypogammaglobulinemic (4.23 ± 0.33 mg/mL) and, at 24 h, the mean serum concentration of IgG was 34.5 ± 1.48 mg/mL, passive immunity transfer was successful. Moreover, colostrum IgG concentrations at 0 h were correlated with serum IgG concentrations at 24 h in buffalo calves. Additionally, TP concentrations were highly correlated with IgG in both colostrum at birth and blood in calves at 24 h. TP is recommended as a reliable indirect parameter to evaluate both colostrum quality and passive immunity transfer in buffalo calves.

Keywords: calf, colostrum, Murrah, neonate, newborn

RESUMO - O objetivo do estudo foi avaliar a transferência de imunidade passiva em bezerros bubalinos sadios. Amostras de colostro de novilhas (sem parto prévio), primíparas e multíparas; e amostras de sangue de seus respectivos bezerros foram coletadas no nascimento, antes da ingestão do colostro, 24h, 48h e 72h após o nascimento para determinação das atividades séricas de gamaglutamiltransferase (GGT) e fosfatase alcalina (ALP); e concentrações séricas de proteína total (PT), imunoglobulinas A (IgA) e G (IgG) e lactoferrina. Os resultados foram analisados como medidas repetidas. As diferenças foram consideradas significativas quando $P \leq 0,05$. Os bezerros nasceram hipogamaglobulinêmicos (4,23±0,33 mg/mL), e às 24h apresentaram concentrações séricas de IgG de 34,5±1,48 mg/mL. A transferência de imunidade passiva foi realizada com sucesso. As concentrações de IgG no

[1] This chapter follows publications guidelines for Arquivo Brasileiro de Medicina Veterinária e Zootecnia – and will be sent to publication.

colostro à 0h foram correlacionadas com as concentrações séricas de IgG às 24h em bezerros. Além disso, as concentrações de PT apresentaram alta correlação com IgG no colostro ao nascimento e no sangue de bezerros às 24h. A PT é recomendada como parâmetro indireto confiável para avaliar a qualidade do colostro e a transferência de imunidade passiva em bezerros bubalinos.

Palavras-chave: bezerro, colostro, Murrah, neonato, recém-nascido

1. Introduction

The water buffalo (Bubalus bubalis) is a species that is important to Asian, Mediterranean, and South American societies because it is essential to the livelihood of many families in developing countries as a source of income and food safety. The most recent reports estimate a world population of 199 million animals, with 193,795,922 heads in Asian countries (FAO, 2019). In South America, Brazil is the largest producer, with 1,381,345 heads, a milk production of 100 million L/year, and an estimated market value of USD 300 million (EMBRAPA, 2018).

As ruminants, buffaloes are dependent on immunoglobulin (Ig) present in the colostrum because they are born agammaglobulinemic or hypogammaglobulinemic (Souza et al., 2019). IgG is responsible for calf immunity during the first month of life and represents 86% of total Igs in buffalo colostrum, with the additional aid of IgA (8%) and IgM (6%) (Dang et al., 2009). The predominance of IgG is due to active and selective receptors in the epithelium of the mammary gland. These same receptors are present in the intestinal epithelial cells of calves and carry IgG through endocytosis in blood circulation (Chaudhary et al., 2018).

The most efficient way to ensure low mortality levels in calves is to verify that passive immunity transfer (PIT) occurs, more specifically, that calves have absorbed at least 20–25 mg/mL of colostral IgG. A failure of PIT (FPIT) prevalence of < 10% is a reasonable goal in ruminants (Meganck et al., 2014). The odds of FPIT are higher when there is no on-farm routine screening, and benchmarking PIT values has been shown to enhance production outcomes as better management practices were adopted on dairy farms (Beam et al., 2009; Atkinson et al., 2017). The measurement of IgG concentrations, both in the

colostrum and blood serum of calves, serve as a tool to evaluate colostrum management.

Despite the importance of the species in world dairy production, the available knowledge regarding PIT in buffaloes remains scarce. Therefore, the aim of this study was to evaluate PIT that occurs in newborn buffaloes by measuring serum concentrations of IgG in both colostrum and calf blood and to evaluate associations among the studied parameters.

2. Material and methods

This research project was evaluated by the Ethical Committee on the Use of Animals of FCAV/UNESP, Jaboticabal Campus (São Paulo, Brazil) and approved under protocol number 17.366/16.

Seventy-two healthy Murrah females (15 heifers without previous calving; 18 primiparous buffaloes; 11 multiparous buffaloes with two or three parities; 15 multiparous buffaloes with four or five parities; and 13 multiparous buffaloes with more than six parities), with an average weight after delivery of 627 ± 91 kg, were examined. They were vaccinated against brucellosis, clostridiosis, hemorrhagic septicemia, bovine viral diarrhea, leptospirosis, and foot and mouth disease. They had free access to Brachiaria spp. pastures and were fed a diet consisting of corn silage, soybeans, and cottonseed twice per day.

Their calves (33 females, 39 males), born from natural breeding via eutocic births and reared on property located in São Paulo state, Brazil (23° 34S, 47°49 W), were also part of this study. After careful observation of all births and immediately after the dams recognized their calves, they were separated, and the birth and weight of the calves were registered. After feeding, the calves' umbilical cords were treated with 2% iodine solution. The calves were free to suckle on their dam during their first five days of life. Only calves that had suckled < 4 h after birth entered the study. Animals underwent thorough daily examination and were considered to be clinically healthy when they did not present any alterations on physical examination (Radostits et al., 2000). Sampling occurred at birth, before they could suckle (0 h), and at 24, 48, and 72 h after birth.

The colostrum samples were pooled from all mammary quarters and stored in 50 mL polypropylene tubes. A 10 mL blood sample from calves was subsequently collected by jugular venipuncture after local antisepsis. A vacuum collection system in siliconized tubes without anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) was used.

Blood samples were clotted at room temperature for 30 min, and serum was collected after centrifugation at 2,000 × g for 10 min and divided into 2.0 mL aliquots and stored in microtubes and maintained at -20 °C until the laboratory tests were performed. For colostrum whey separation, renin solution was added (Coalho Estrella, Chr. Hansen Brasil Ind. and Com. LTDA, Valinhos, Brazil) in an amount corresponding to 5% of the volume of milk secretion. The samples were placed in a 37 °C water bath for 20 min until formation and retraction of the clot. Subsequently, samples were centrifuged at 5,000 × g for 20 min in a refrigerated (4 ºC) centrifuge. After centrifugation, 10 mL of serum was aspirated and stored in microtubes, then frozen at -20 °C until analysis.

Serum activities of gammaglutamyltransferase (GGT) (modified Szasz method) and alkaline phosphatase (ALP) (modified Bowers and McComb method) and serum concentrations of total protein (TP) (biuret method) were analyzed using a semiautomatic spectrophotometer (Labquest, Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil) with light of the appropriate wavelength for each test.

Protein fractionation of colostrum and serum samples were determined using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the technique described by Laemmli (1970). The concentrations of these proteins were determined using computerized densitometry (Shimadzu CS9301, Tokyo, Japan). As a reference, a marker solution with different molecular weights was used in addition to the purified bovine IgG protein (Sigma, St Louis, MO, USA).

To observe the effects of treatment (dam colostrum or calf blood) throughout the experimental period (0, 24, 48, and 72 h after calving) and the respective interactions (treatment × time), the parameters were analyzed as repeated measures using the MIXED procedure in SAS version 9.4 (SAS/STAT,

SAS Institute Inc., Cary, NC, USA). In cases wherein the premise of specificity was not met ($P < 0.05$), the probabilities of time (P value of time) and interactions of treatment and time (P value for treatment × time) were corrected using the Greenhouse-Geisser epsilon formula. Comparison between treatments at each time point (P value) was performed using the least-squares means (LS means) test following repeated measures analysis.

Correlation analysis among all response variables was performed using the CORR RANK procedure in SAS. Relationships among the studied parameters were determined from these correlations. To predict 24-h-old buffalo calf IgG or colostrum IgG (dependent variables), non-polynomial simple regression equations were formulated using other responses as independent variables (colostrum IgG, colostrum TP, and 24-h-old calf TP). For this, the GLM procedure of the SAS program was used, using the LS means methodology.

The regression and correlation coefficients obtained were considered high only when determination coefficients ($R2) \geq 0.70$ and correlation coefficients ($r) \geq 0.50$. P was considered to be statistically significant at ≤ 0.05. Graphics were created using Sigmaplot version 12.0 (Systat Software GmbH, Erkrath, Germany).

3. Results and discussion

The calves had an average weight of 38.0 ± 5.55 kg. Serum concentrations of IgG, IgA, lactoferrin, and TP and serum activities of GGT and ALP in the colostrum and blood of calves are shown in Figure 1.

PIT is determined according to colostrum quality and calf absorption. The moment of colostrum intake, method of administration, and volume of colostrum are linked to calf absorption, whereas nutrition, breed, calving interval, dry-off period, and vaccination are determinants of colostrum quality. Therefore, IgG concentrations in both colostrum and calf sera should be estimated regularly to test compliance with colostrum management (Meganck et al., 2014; Patel et al., 2014).

The most common methods to assess the PIT status in domestic animals, measuring direct IgG concentrations, are single radial immunodiffusion (SRID), used as the gold standard, and enzyme-linked immunosorbent assay (ELISA), even if these methods are not feasibly correlated (Dunn et al., 2018). SDS-PAGE, refractometry, and sodium sulfite or zinc sulfate turbidity test are used to estimate serum IgG concentration based on the overall protein concentration (Gapper et al., 2007). Moreover, there are new on-farm methods, such as the split trehalase IgG assay (STIGA), still undergoing validation testing (Drikic et al., 2018).

The mean serum concentration of IgG in the colostrum of dams was 71.4 ± 2.81 mg/mL on first milking, which was a high value considering that 50 mg/mL is used as the standard for good quality colostrum in bovine cattle, as no standard for buffaloes is available, (Patel et al., 2014). This result was higher than those reported for Egyptian buffaloes using SRID (33.20 mg/mL) (El-Fattah et al., 2012) and Murrah buffaloes using indirect ELISA (54 mg/mL, 51.7 ± 5.99 mg/mL, and 57.9 ± 5.71 mg/mL) by Dang et al. (2009), Chaudhary et al. (2018) and Verma et al. (2018), respectively. After calving, IgG concentration decreases as colostrum finishes turning into milk at approximately the fifth day after birth (El-Fattah et al., 2012).

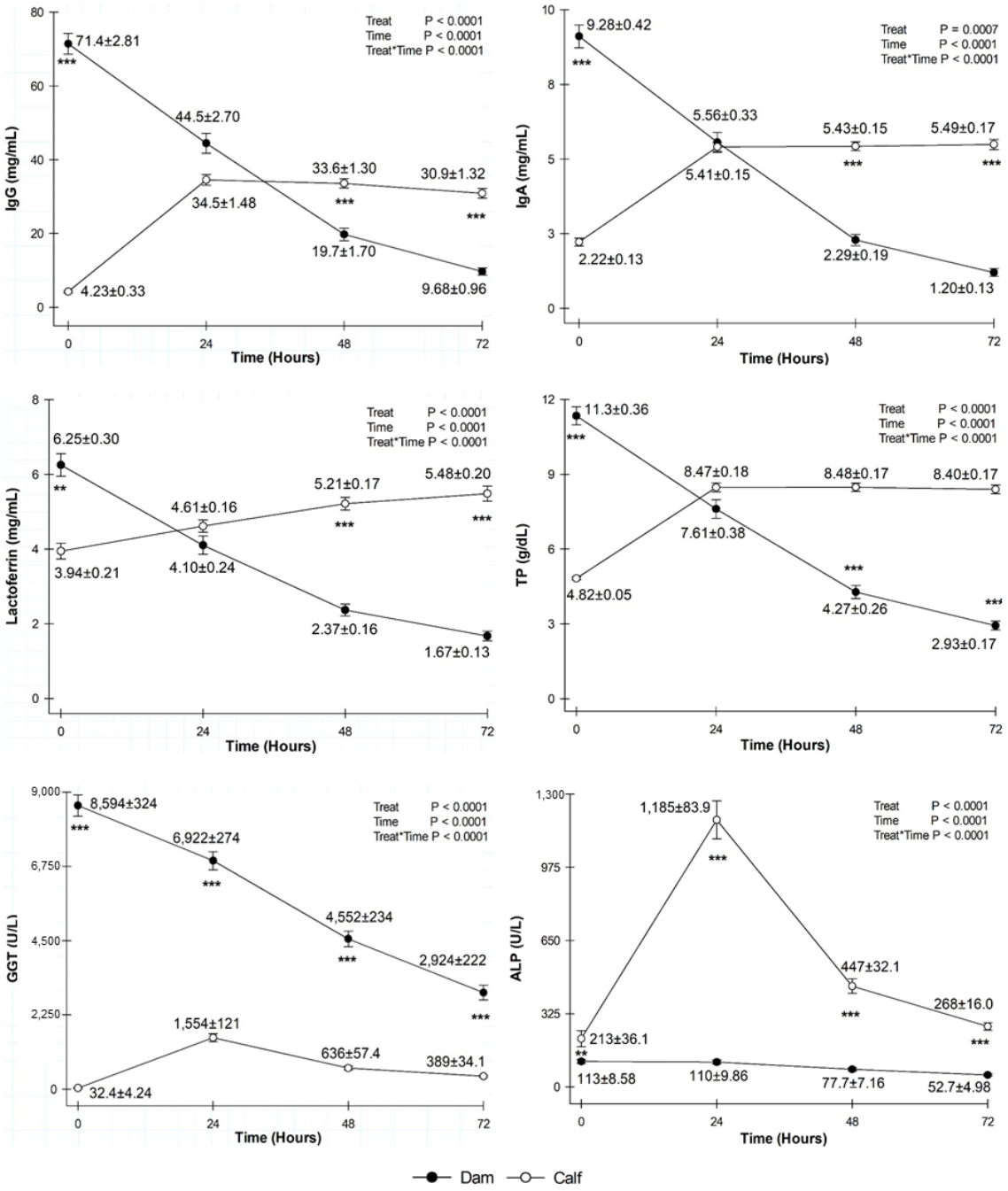

Figure 1. Mean ± standard deviation of serum concentrations of immunoglobulin G (IgG), IgA, lactoferrin, and total protein (TP) and serum activities of gammaglutamyltransferase (GGT) and alkaline phosphatase (ALP) in the colostrum (black circles) and serum of calves (white circles) before colostrum intake and at 0 h, 24 h, 48 h, and 72 h. *P ≤ 0.05; **P < 0.01; ***P < 0.0001.

The results of our study can be linked to the vaccination schedule of the farm, which must be responsible for the high IgG concentrations in colostrum derived from the dam's bloodstream. Additionally, all buffaloes had good nutrition and had their dry-off period respected, which is important so the mammary gland can recover from the previous lactation. This is essential in primiparous dams, which experience significant physiological changes during their first lactation. Moreover, the division between categories enabled the proper feeding of heifers. Without the dominance of older dams, heifers could eat an amount adequate for growth, calf development and their immune system status, culminating in high serum concentrations of IgG in colostrum.

Calves were born hypogammaglobulinemic (4.23 ± 0.33 mg/mL) and after suckling colostrum; at 24 h, serum IgG concentrations increased, to an average of 34.5 ± 1.48 mg/mL. Similar values have been reported at 24 h in Mediterranean buffalo calves (31.0 ± 2.4 mg/mL (Mastellone et al., 2011) and descendants of Murrah heifers at 24 h (35.3 ± 8.58 mg/mL (Souza et al., 2019). and, moreover, higher than those measured by indirect ELISA at 6–12 h and 12–18 h after first feeding (11.7 ± 0.75 mg/mL (Chaudhary et al., 2016) and 11.2 ± 0.7 mg/mL (Verma et al., 2018).

The high serum concentrations of IgG in calf blood in this study can be attributed to the short time between birth and first feeding (< 4 h after birth). As shown in Figure 1, there is a point of inflexion at 24 h that is common for both colostrum and calf blood serum concentrations, with the IgG concentrations in calf blood surpassing the amount of IgG in colostrum after 48 h. Because calves are allowed to nurse the dam freely, the volume of colostrum ingested by the calf is not limited by human workforce or proper colostrum management, resulting in high serum concentrations of IgG that will endure until the first month of age (Souza et al., 2019).

The correlation between dam colostrum parameters at birth and calf blood parameters at 24 h are summarized in Table 1. A correlation index of 0.46357% ($P < 0.0001$) was found within dam colostrum IgG at parturition and serum concentrations of IgG at 24 h in calf sera.

Table 1. Correlation between colostrum parameters at birth and serum concentrations parameters of buffalo calves at 24h.

	IgGDam	IgADam	LactDam	TPDam	GGTDam	ALPDam	IgGCalf	IgACalf	LactCalf	TPCalf	GGTCalf	ALPCalf
IgGDam												
r	1.00000											
P												
IgADam												
r	0.88922	1.00000										
P	<.0001											
LactDam												
r	0.35222	0.33584	1.00000									
P	0.0040	0.0055										
TPDam												
r	0.97376	0.85897	0.42306	1.00000								
P	<.0001	<.0001	0.0004									
GGTDam												
r	0.35266	0.19275	0.25664	0.37227	1.00000							
P	0.0046	0.1210	0.0423	0.0027								
ALPDam												
r	-0.01078	0.09610	0.29534	0.04029	0.22487	1.00000						
P	0.9310	0.4287	0.0153	0.7462	0.0652							
IgGCalf												
r	0.46357	0.38570	-0.00623	0.38910	0.14147	0.02769	1.00000					
P	<.0001	0.0011	0.9604	0.0012	0.2535	0.8174						
IgACalf												
r	0.12043	0.19029	-0.12646	0.08732	-0.08366	0.11745	0.43874	1.00000				
P	0.3355	0.1173	0.3116	0.4857	0.5009	0.3258	0.0001					
LactCalf												
r	-0.19010	-0.16907	-0.09991	-0.19359	0.04654	-0.14275	0.12398	0.14295	1.00000			
P	0.1263	0.1649	0.4248	0.1194	0.7084	0.2316	0.2995	0.2310				
TPCalf												
r	0.39218	0.37178	-0.07874	0.31104	0.07702	-0.02464	0.92647	0.48188	0.31736	1.00000		
P	0.0011	0.0017	0.5297	0.0110	0.5356	0.8372	<.0001	<.0001	0.0066			
GGTCalf												
r	0.16585	0.11808	0.15167	0.14650	0.49548	0.06960	0.39202	0.01978	0.19161	0.34779	1.00000	
P	0.1867	0.5670	0.2278	0.2442	<.0001	0.5670	0.0008	0.8709	0.1121	0.0032		
ALPCalf												
r	0.34998	0.21762	0.08413	0.29454	0.21756	-0.02902	0.26533	0.02456	-0.35733	0.04735	0.17515	1.00000
P	0.0040	0.0725	0.5018	0.0164	0.0770	0.8102	0.0253	0.8389	0.0022	0.6950	0.1470	

It has been established that PIT evaluation is better performed between 24 h and 72 h after first feeding. However, measurements within one week or even 10 days after birth can be performed with reliable confidence (Patel et al., 2014; Wilm et al., 2018). A cut-off point of 10 mg/mL for IgG is recommended to determine PIT in dairy bovine calves (Weaver et al., 2000); however, a more realistic standard of 20–25 mg/mL has recently been established for dairy calves based on the improvements made in colostral feeding practices in the dairy industry (Chigerwe et al., 2015). Unfortunately, there is no cut-off point for PIT in buffalo calves.

Nevertheless, a more efficient PIT means a better average daily gain (ADG) and heavier calves at 30 days in buffaloes (Mastellone et al., 2011), with the same positive correlation between serum IgG levels and ADG reported in pre-weaned Holstein calves (Elsohaby et al., 2019). It is important to strive for higher PIT levels because it clearly enhances production efficiency on dairy farms, contrary to a previous report that there is no benefit in surpassing the recommended IgG threshold for avoiding FPIT (Weaver et al., 2000).

The mean IgA concentration in dam colostrum soon after birth was 9.10 ± 3.89 mg/mL. The present study had higher values at 24 h than those reported by Dang et al (2009) in Murrah buffaloes using ELISA (3.22 mg/mL). This difference is probably due to the data sample (n = 72 versus n = 8 dams) and method of quantification (SDS-PAGE versus ELISA).

Calves were born with low serum levels of IgA (2.23 ± 0.14 mg/mL). After feeding, at 24 h, newborns exhibited an average IgA level of 5.41 ± 0.15 mg/mL. IgA acts to prevent infections through agglutination of microorganisms, binding to the intestinal wall receptors and, together with IgG, is essential for providing neonates with immunological protection during at least the first 2 to 4 weeks of life (Chase et al., 2008; Tizard, 2009).

The mean lactoferrin concentration in dam colostrum was 6.25 ± 0.30 mg/mL on first milking, higher concentrations than those of Egyptian dams (1.08 mg/mL) soon after birth (El-Fattah et al., 2012). Lactoferrin is present in buffalo milk in lower quantities 0.05–3.40 mg/mL (El-Salam and El-Shibiny, 2011) and is an indicator of the health of mammary glands. It acts as an important

immunomodulator because it binds to iron molecules, impeding their availability to bacteria because they bind to cell-surface receptors and facilitate iron absorption (Tizard, 2009).

Serum concentrations of lactoferrin in calves exhibited an increase after first feeding, with an average of 4.61 ± 0.16 mg/mL and continued increasing until the third day (5.48 ± 0.20 mg/mL), as it continued to be absorbed different from what occurs with Igs (Fig.1). Lactoferrin, when absorbed and in plasma, is called “transferrin” and is responsible for iron transportation. In addition, it has other functions including antiviral and antibacterial activities, and also acts as a growth factor (Tizard, 2009).

We found high correlation indexes between both colostrum IgG and TP at the first milking (0.97376 [$P < 0.0001$]), and between serum concentrations of IgG and TP in calf sera at 24 h after birth (0.92647 [$P < 0.0001$]). Determination indexes are presented in scatter plots in Figure 2.

TP is a reliable indirect parameter used to evaluate PIT because TP concentrations have high sensitivity and specificity for the detection of FPIT. Due small variation in serum albumin concentrations in newborns, the increase in TP concentrations is almost exclusively due to the absorption of Igs present in colostrum (Hogan et al., 2015).

Dam colostrum had an average TP concentration of 11.3 ± 2.4 g/dL soon after parturition. TP dynamics, presented in Fig. 1, show a turning point both in colostrum and calf blood at 24 h, with the TP concentrations in calf sera surpassing those from colostrum and without a significant difference between them ($P \leq 0.05$). Mean serum TP concentration in calves before colostrum feeding was 4.82 ± 0.05 g/dL and, at 24 h of age, reached an average concentration of 8.47 ± 0.18 g/dL, which was above the recommended threshold for an efficient PIT in dairy bovine calves at 24 h (5.8–6.3 g/dL) and consistent with serum TP concentrations reported in buffalo calves born from multiparous buffaloes (Chigerwe et al., 2015; Souza et al., 2019).

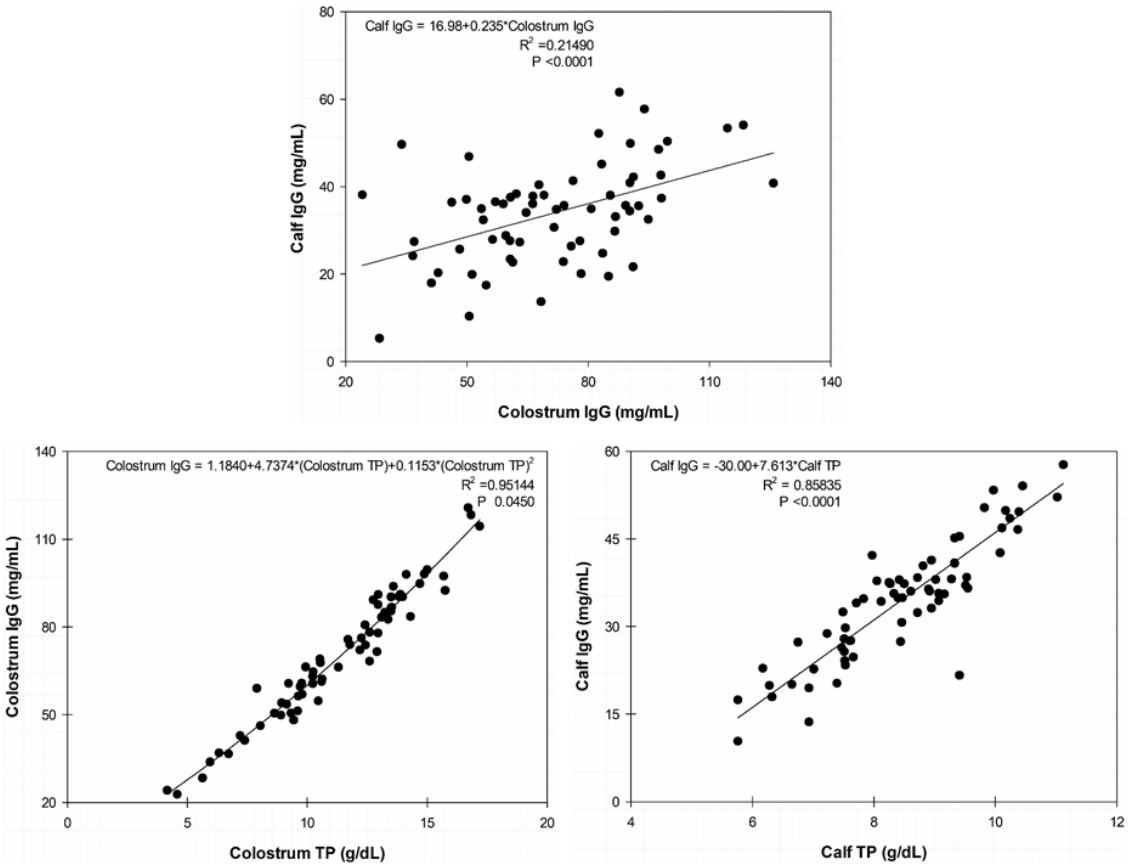

Figure 2. Scatter plots of calf immunoglobulin (Ig)G at 24 h × colostrum IgG at 0 h; colostrum IgG × colostrum total protein (TP) at 0 h; and calf IgG × calf TP at 24 h. The line represents the tendency of the data determined by simple linear regression on calf blood serum and quadratic regression on dam colostrum.

In water buffaloes, GGT determines the availability of amino acids for milk protein synthesis during lactation, and is present in great quantities during colostrogenesis, accumulating until first milking. In this manner, it has positive correlation (86%) with IgG values measured electrophoretically and is recommended to evaluate colostrum quality from buffalo dams (Lombardi et al., 2001; Pero et al., 2006). In addition, GGT levels demonstrated the best balance between sensitivity and specificity when compared with IgG measured by ELISA using SRID as gold standard (Hogan et al., 2015).

Blood serum activities of GGT in calves exhibited an increase between 0 h and 24 h (32.4 ± 4.24 to 1554 ± 121 U/L) following the ingestion of the enzyme present in high quantities in colostrum at the time of birth (8594 ± 324 U/L). In the

following days, serum activities in calf sera decreased, as GGT was degraded over time by the calf intestine (Fig 1).

A correlation of 0.49548 (P<0.0001) was found between colostrum serum activities of GGT at first milking and blood serum activities in calves 24 h after birth, which confirms the origin of high serum activities of GGT in calves in the first week of life (Souza et al., 2019).

The serum activity of ALP in calf blood was low soon after birth and increased at 24 h, with an average of 1,185 ± 83.9 U/L. This could not be due to absorption of ALP from colostrum because it had low levels since the first sampling (113 ± 8.58 U/L).

There was no correlation between the serum activity of ALP on first milking and calf blood at 24 h. Moreover, a weak correlation was found between ALP content and serum concentrations of IgG at 24 h (0.26533 [P< 0.05]).The non-correlation of this enzyme with IgG concentrations has been previously reported (Lombardi et al., 2001).

The increase in serum activity of ALP might be due to the isoenzyme of bone origin because there is an increase in activity of bone isoenzyme ALP in animals with high osteoblastic activity, as in neonates (Kaneko et al., 2008) (Kaneko et al., 2008).

4. Conclusions

PIT was successfully achieved in the studied calves. Colostrum from buffalo dams had high IgG concentrations before first milking, which resulted in high IgG concentrations in the calf blood 24 h after birth. Moreover, concentrations of TP served as reliable indirect parameters to evaluate colostrum quality and PIT in buffalo calves. ALP had no significant correlation with IgG in both colostrum at 0 h and calf sera at 24 h; thus, this enzyme is not recommended for assessing PIT.

Calves had IgG concentrations well above the utilized cut-off point in dairy calves. We emphasize the importance of a vaccination schedule and proper nutrition for dams. Additionally, it is important for calves to nurse directly and freely from the dam. The presence of the dam with the newborn in the first days after calving is also important.

5. Acknowledgements

The authors acknowledge the contribution of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) in the scholarship granted - Finance Code 001. And also the Paineras do Ingaí farm in which the study was developed.

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CHAPTER 3 – Relationship between productive performance and serum concentrations of immunoglobulin G of Murrah calves allowed to nurse the dam[2]

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ABSTRACT - The aim of this study was to evaluate the influence of serum concentrations of immunoglobulin G (IgG) at 24hs on growth performance in the first 90 days of age of Murrah buffalo calves. Blood samples were taken at 24h after birth for determination of IgG using polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) and weight was measured following each week after birth until three months of age. Results were evaluated by simple linear regression to compare weight and average daily gain (ADG) as dependent variables and serum concentrations of IgG at 24h as independent variable. Calves had serum concentrations of IgG at 24h of 34.5±1.25 mg/mL and an average weight at birth of 38.0±5.54 Kg, and a weight and ADG with 30, 60 and 90 days of age respectively as follows: 49.2±6.30 Kg; 61.7±8.14 Kg; 75.9±10.3 Kg and 0.363±0.139 Kg/d; 0.391±0.101 Kg/d; 0.421±0.091 Kg/d. No significant association of serum concentrations of IgG at 24h in the weight or ADG was detected. Production outcomes of calves on the first 90 days of age on buffalo calves are more influenced by the dam's nutrition, genetic heritable characteristics and good health management of the calf.

Keywords: calf, colostrum, Murrah, neonate, newborn

RESUMO - O objetivo deste estudo foi avaliar a influência das concentrações séricas de imunoglobulina G (IgG) às 24 horas no desempenho produtivo nos primeiros 90 dias de bezerros Murrah. Amostras de sangue foram coletadas 24 horas após o nascimento para determinação da IgG por eletroforese em gel de poliacrilamida contendo dodecilsulfato de sódio (SDS-PAGE) e o peso foi medido semanalmente após o nascimento até os três meses. Os resultados foram avaliados por regressão linear simples comparando as concentrações séricas de IgG às 24h como variável independente e o peso e o ganho de peso diário (GPD) como variáveis dependentes. Os bezerros apresentaram concentrações séricas de IgG às 24h de 34,5±1,25 mg/mL e um peso ao nascimento de 38,0±5,54 Kg. O peso e GPD com 30, 60 e 90 dias de idade, foram: 49,2±6,30 Kg; 61,7±8,14

[2] This chapter follows publications guidelines for Arquivo Brasileiro de Medicina Veterinária e Zootecnia – and will be sent to publication.

Kg; 75,9±10,3 Kg e 0,363±0,139 Kg/d; 0,391±0,101 Kg/d; 0,421±0,091 Kg/d respectivamente. Não foi detectada associação significativa das concentrações séricas de IgG às 24h com o peso ou GPD durante o período do estudo. Os resultados produtivos de bezerros nos primeiros 90 dias de idade em bezerros bubalinos são mais influenciados pela nutrição da mãe, características genéticas herdáveis e bom manejo sanitário dos bezerros.

1. Introduction

The water buffalo (*Bubalus bubalis*) production is made among all continents, with more emphasis on Asia (195,772,907 heads) and South America (1,382,130 heads) (FAO, 2019). On Brazil with 1,381,395 heads a milk production of 100 million L/y generates an economic impact of approximately US$ 300 million (EMBRAPA, 2018).

One critical production point of buffaloes is the neonatal phase that is comprised of the first month of living with high mortality rates reaching from 16% to 36% (Shivahre *et al*., 2014; Kharkar *et al*., 2019). As buffaloes are born agammaglobulinemic or hypogammaglobulinemic, they are dependent from the immunoglobulins (Ig) present in colostrum, which will give them the immunity to survive until the end of neonatal period, when they will activate they own immune response (Souza *et al.*, 2019).

The most efficient way to ensure low mortality levels in dairy calves is to make sure that passive immunity transfer (PIT) is done within the first hours after birth, meaning that calves have at least 20–25 mg/mL of blood serum concentrations of immunoglobulin G (IgG) at 24h (Chigerwe *et al*., 2015). Benchmarking individual PIT values on each farm has been proved to enhance production outcomes as better management practices can be adopted to ensure PIT (Beam *et al*., 2009; Atkinson *et al*., 2017). Also, high concentration of Igs had been proven to result in higher growth performance values (Mastellone *et al*., 2011; De Paula *et al*., 2019).

Despite the importance of the species to world dairy production, the available knowledge about PIT values influence on performance traits of buffaloes is still scarce. We believe that calf body development is directly related to the quality of passive immunity transfer, and that weight performance predicts the quality of passive immunity transfer. Therefore, the aim of this study was to

evaluate the influence of serum concentrations of IgG at 24h in average daily gain and weight values at 30, 60, and 90 days of age of buffalo calves.

2. Material and methods

The research project was evaluated by the Ethical Committee on the Use of Animals (CEUA) of FCAV/UNESP, Jaboticabal campus, and approved under protocol 17.366/16.

The study was carried out between October of 2014 and August 2015, in a farm located São Paulo State (23°34 S, 47°49 W). Seventy-six healthy Murrah buffalo calves from both sexes (36 females and 40 males) were born from natural mating, via eutocic births, from 17 heifers without previous calving; 19 primiparous buffaloes with one calving; 11 multiparous buffaloes with two or three parities; 15 multiparous buffaloes with four or five parities and 14 multiparous buffaloes with more than six parities with an average weight after parturition of 627±91 Kg.

After suckling colostrum directly from their mothers, calves umbilical cords were treated with 2% iodine solution. Calves stayed on maternity pen, and were free to suckle on their mothers during their first five days of age. Then calves were raised in a collective pen, and were fed milk from a mammary quarter two times a day during the milking of their dams. In addition to the milk diet, calves had free access to *Brachiaria spp.* Pastures, corn silage and a ration composed of soybeans and cottonseed.

Calves went through daily examination and considered clinically healthy when they did not present any alterations in physical examination (Radostits *et al*., 2000). When animals showed any symptoms or achieved temperature levels ≥39.6°C, they received proper treatment with antibiotics and anti-inflammatory.

All calves received treatment against endoparasites with albendazole sulfoxide 13.6% - (Ricobendazole 13,6%, Chemitec Ltda., São Paulo, Brazil) or albendazole 10% (Biozen, Biofarm, São Paulo Brazil) with seven and 21 days of age. Pulverization of fipronil 1% (Topline Red, Merial, Campinas, Brazil) was made if calves had a tick infestation, also an application of abamectin 1% (Abamec LA, Ourofino SA, Cravinhos, Brazil) or doramectin 1% (Dectomax,

Zoetis, Campinas, Brazil) was made with two months of age. All animals were vaccinated against pasteurellosis (Tifopasteurina, Ceva, Paulínia, Brazil) and clostridiosis (Polistar, Vallé, Montes Claros, Brazil) with two months of age. All calves were characterized as negative for rotavirus on electrophoresis tests on polyacrylamide gel (PAGE).

A 10 mL blood sample of the calf was made by jugular venipuncture after local antisepsis at 24h of age with a vacuum collection system in siliconized tubes without anticoagulant (Vacutainer, Bencton Dickinson, Franklin Lakes, USA). Blood samples were clot at room temperature for 30 min and serum was collected after centrifugation at 2,000 x g for 10 minutes, and 2.0 mL aliquots of serum were separated and stored in microtubes, previously identified and maintained at -20°C until laboratory tests.

Serum concentrations of immunoglobulin G were determined by protein fractionation of serum samples using polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE), according to the technique proposed by Laemmli (1970). The concentrations of proteins were determined by computerized densitometer (Shimadzu CS-9301 PC, Tokyo, Japan) using a reference, a marker solution with different molecular weights in addition to the purified bovine IgG protein (Sigma, St Louis, MO, USA).

Calves were weighted at birth and subsequently every week until the third month of age. Average daily gain (ADG) was calculated by the difference between the weight at 30, 60 and 90 days of age and the birth weight, and divided by the number of days passed.

Continuous variables were presented as the mean ± standard error of the mean (mean ± SEM). Correlation analysis among all response variables was performed using the CORR RANK procedure of SAS® version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC, USA).

Relationships among the IgG at 24h, weight at 24h, 30, 60 and 90d, ADG up to 30, 60 and 90d were determined from these correlations. For prediction of weight at birth, 30, 60 and 90 days of age, as well as average daily gains up to 30, 60 and 90 days of age (dependent variables), non-polynomial simple regression equations were formulated using 24-hour-old buffalo calf IgG,

(independent variable). For this, the GLM procedure of the SAS program was used, using the least squares methodology.

For the regressions and correlation coefficients obtained, they were considered high only when the determination coefficients (r^2) were ≥0.70 and correlation coefficients (r) were ≥0.50. P value was considered significant when ≤ 0.05. The graphics were created using Sigmaplot 12.0 software programs (Systat Software GmbH, Erkrath, Germany).

3. Results and discussion

Calves had serum concentrations of immunoglobulin G (IgG) at 24h of 34.5±1.25 mg/mL. These values are above the thresholds of 20-25 mg/mL recommended to bovine dairy calves and those of 27 mg/mL for beef calves (Dewell *et al*. 2006; Chigerwe *et al*., 2015). Those are similar to previous reports on buffalo calves at 24h: 31.0±2.4/35.3±8.58 mg/mL and synonymous of low calf mortality, reduced number of disease episodes and heavier calves (Mastellone *et al*., 2011; Elsohaby *et al*., 2019; Souza *et al*., 2019).

Birth weight average (38.0±5.54 Kg) was higher than those of Mediterranean calves from Italy (35.4±3.9 Kg); Murrah calves (32.6±0.42; 32.4 ± 0.30 Kg) from India ; Nili-Ravi calves from Pakistan (35.86±4.30 Kg); and Surti calves from India (24.60±0.18 Kg; 26.2±3.9 Kg) and Mediterranean calves from Turkey (26.95±0.25 Kg) (Thiruvenkadan *et al.*, 2009; Mastellone *et al.*, 2011; Akhtar *et al.*, 2012; Gupta *et al.*, 2012; Pandya *et al.,* 2015; Ugurlu *et al*., 2016; Singh *et al*., 2019).

Higher values at birth are important as heavier animals gain more weight easier in the following phases as they are more prone to compete for food. Also, these animals with proper nutrition, achieve physiological and reproductive maturity earlier than lighter animals (Cruz-Cruz *et al.*, 2019)

Buffaloes had an average weight at 30 days of 49.2±6.30 Kg, below to those from Mediterranean calves of (56.4±4.4 Kg) (Mastellone *et al.*, 2011).

An average weight of 61.7±8.14 Kg at 60 days was found on this study, similar the one reported on Nili-Ravi calves (62.7 Kg) raised on similar fashion as the ones from our study (Abbas *et al.*, 2017).

Buffaloes weight at 90 days (75.9±10.3 Kg) were lower when compared to Nili-Ravi calves (83.5 Kg) under similar conditions (Abbas *et al.,* 2017); and higher than those reported on Murrah calves (62.0±0.65 Kg), probably due to the early period in which the study was done (1990-2004); Surti calves (49.93±0.36 Kg) and Nili-Ravi calves (66.12±9.16 Kg) under more extensive raising conditions (Thiruvenkadan *et al.*, 2009; Akhtar *et al.*, 2012; Pandya *et al*., 2015).

According to the literature weight of Murrah calves is not affected significantly by season of birth. Whereas, sex of the calf affects weight at birth ($P<0.05$) and 90 days weight ($P<0.01$). With male calves been heavier since they are born, and that difference rising as animals get older. Number of parities of the dam also has a significant effect on ($P<0.01$) those same measures, as calves from first parity dam being lighter (Thiruvenkadan *et al.*, 2009).

Weight of the dam at the time of birth has a significant effect on birth weight ($P<0.05$) of Nili-Ravi calves. With heavier dams (500-550 Kg) giving birth to heavier calves and lighter dams (330-400 Kg) giving birth to lighter calves (Akhtar *et al*., 2012). In our study mean weight of dams after parturition was of 627±91 Kg and we ruled out this factor as dams were all considered heavy.

The ADG of calves in the present study were higher throughout the study (0.363±0.139 Kg/d; 0.391±0.101 Kg/d; 0.421±0.091 Kg/d) 30, 60 and 90 days of age than pre-weaning ADG average of Nili-Ravi (0.316±0.08 Kg/d), and lower than Nili-Ravi calves (0.502 Kg/d) raised on similar fashion as the ones from this study; and of Mediterranean calves weaned at birth in a more intensive production (0.726±0.187 Kg/d) (Mastellone *et al.*, 2011; Abbas *et al*., 2017).

Higher ADG average is reported in buffalo calves that are allowed to nurse the dam when compared to weaned calves (0.506 vs. 0.438 Kg/day). Also immune status, health, behavior and oxidative stress of natural suckling calves had better outcomes when compared to that of weaned calves. (Singh *et al.*, 2019).

On Brazil the majority of buffalo farms where dairy production is done uses the natural suckling as raising methods for calves. That is due to the let-down of milk to udder by the calf stimuli. In addition, there is also the reunion of the calf with the dam after milking, where calf sucks the residual milk left in the udder.

Also, calves that suckle directly from their dams have a slower rate of feeding, mixing saliva and digestive enzymes properly, resulting in better absorption of immunoglobulins and nutrients content in colostrum as well as milk (Singh *et al.*, 2019).

The correlations between the studied variables are presented in Table 1.

Table 1. Correlation coefficients between serum concentrations of immunoglobulin G at 24h and weight at birth, 30, 60 and 90 days, and average daily gain up to 30, 60 and 90 days in buffalo calves.

	IgG[a]	BW[b]	W30d[c]	W60d[d]	W90d[e]	ADG30[f]	ADG60[g]	ADG90[h]
IgG								
r	1.00000							
P								
BW								
r	-0.08948	1.00000						
P	0.4681							
W30d								
r	0.04147	0.73836	1.00000					
P	0.7370	<0.0001						
W60d								
r	-0.18476	0.68636	0.82160	1.00000				
P	0.1315	<0.0001	<0.0001					
W90d								
r	-0.16073	0.63198	0.79076	0.87597	1.00000			
P	0.1904	<0.0001	<0.0001	<0.0001				
ADG30								
r	0.18243	0.13058	0.53421	0.36616	0.39022	1.00000		
P	0.1365	0.2885	<0.0001	0.0021	0.0010			
ADG60								
r	-0.17240	0.03222	0.42912	0.74900	0.62809	0.62217	1.00000	
P	0.1598	0.7942	0.0003	<0.0001	<0.0001	<0.0001		
ADG90								
r	-0.14547	0.13771	0.49584	0.65968	0.85463	0.58621	0.78114	1.00000
P	0.2365	0.2628	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

a – Immunoglobulin G (mg/mL); b – Birth Weight (Kg); c – 30d Weight (Kg); d – 60d Weight (Kg); e – 90d Weight (Kg); f – Average Daily Gain at 30d (Kg/d); g – Average Daily Gain at 60d (Kg/d); h – Average Daily Gain at 90d (Kg/d)

This work found significant correlation between birth weight and weight at 30, 60 and 90 days of age (P<0.0001). Corroborating what has been previously reported (Thiruvenkadan *et al.*, 2009).

Association between serum concentrations of IgG at 24h and weight during the period studied can be seen in Figure 1.

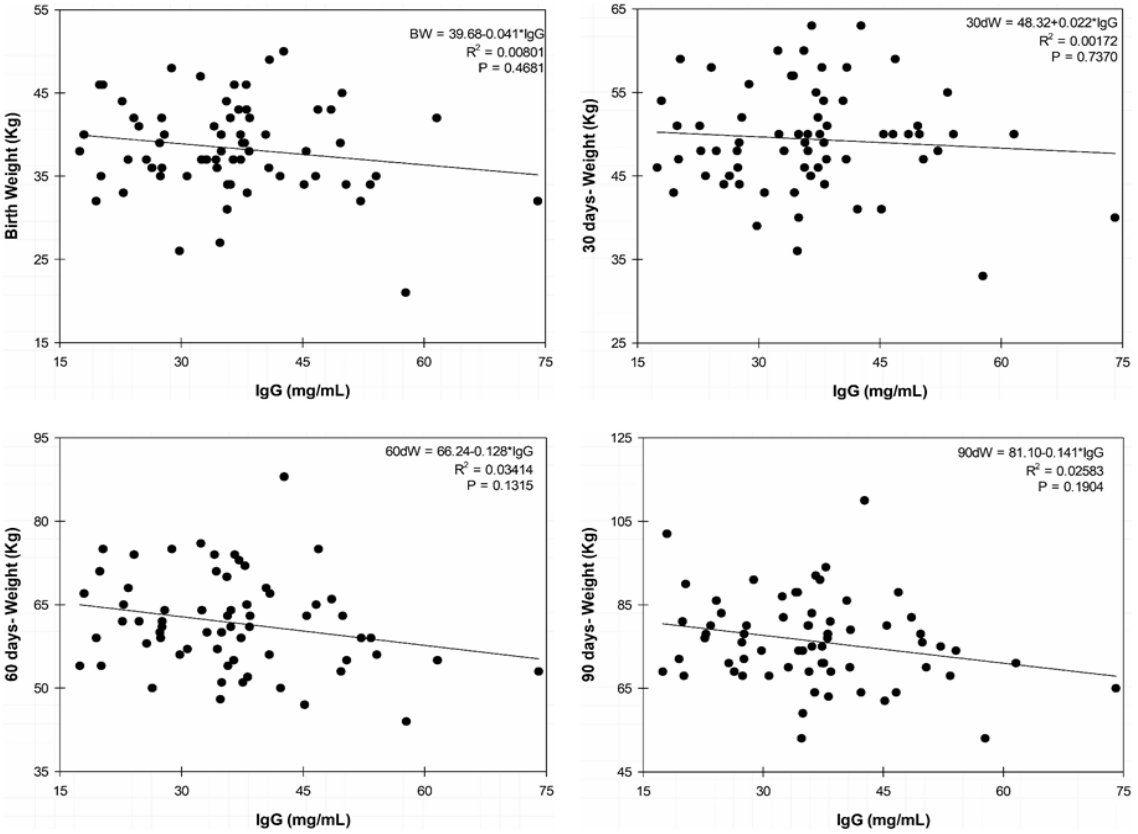

Figure 1. Scatter plots of weight at birth, 30, 60 and 90 days of age versus serum concentrations of immunoglobulin G (IgG) at 24h of buffalo calves that nurtured colostrum directly from the dams. The line represents the tendency of the data determined by simple linear regression.

There was no association between IgG at 24h and birth weight, and weight of the calves on 30, 60 and 90 days of age in this study, different to what was stated previously in buffalo calves, where a significant (P<0.001) negative association was detected between birth weight and IgG (Mastellone *et al*., 2011).

Absence of relationship between IgG at 24 and birth weight was also reported on healthy dairy lambs allowed to nurse from their dams (Gokce *et al.*, 2013).

An association between body weight and low levels of IgG, determined by serum total protein, was reported in dairy heifer calves were animals who did not achieved 5.2-5.9 g/dL had lower body weight during the first three months of age when compared to animals above that threshold (Windeyer *et al*., 2014).

Passive immunity transfer (PIT) is determined by colostrum quality and calf absorption. The time of ingestion, method of administration, and volume of colostrum are linked to calf absorption. Whereas nutrition, breed, calving interval,

dry-off period and vaccination are determinants of colostrum quality. Therefore, IgG concentrations on both colostrum and calf sera should be estimated regularly to test the compliance of the colostrum management (Meganck *et al*., 2014; Patel *et al*., 2014).

The absence of correlation between IgG at 24h and weight can be explained by the fact that the studied calves were all feed <4h after birth. As it is clear that early postnatal colostrum intake is important for weight gain in the neonatal phase, as demonstrated previously on calves that were fed 0-2h after birth showed better performance when compared to calves fed >6h after birth (Zanker *et al.*, 2001).

As calves were allowed to nurse the dam freely, the volume of colostrum ingested by the calf is not limited by human workforce or proper colostrum management, resulting in high serum concentrations of IgG that will endure until the first month of age of the calf (Souza *et al.*, 2019), what makes the difference when compared to calves weaned early that may have lower weights in the first months of living (Cruz-Cruz *et al.*, 2019)

Association between serum concentrations of IgG at 24h and ADG at 30, 60 and 90 days of age can be seen in Figure 2.

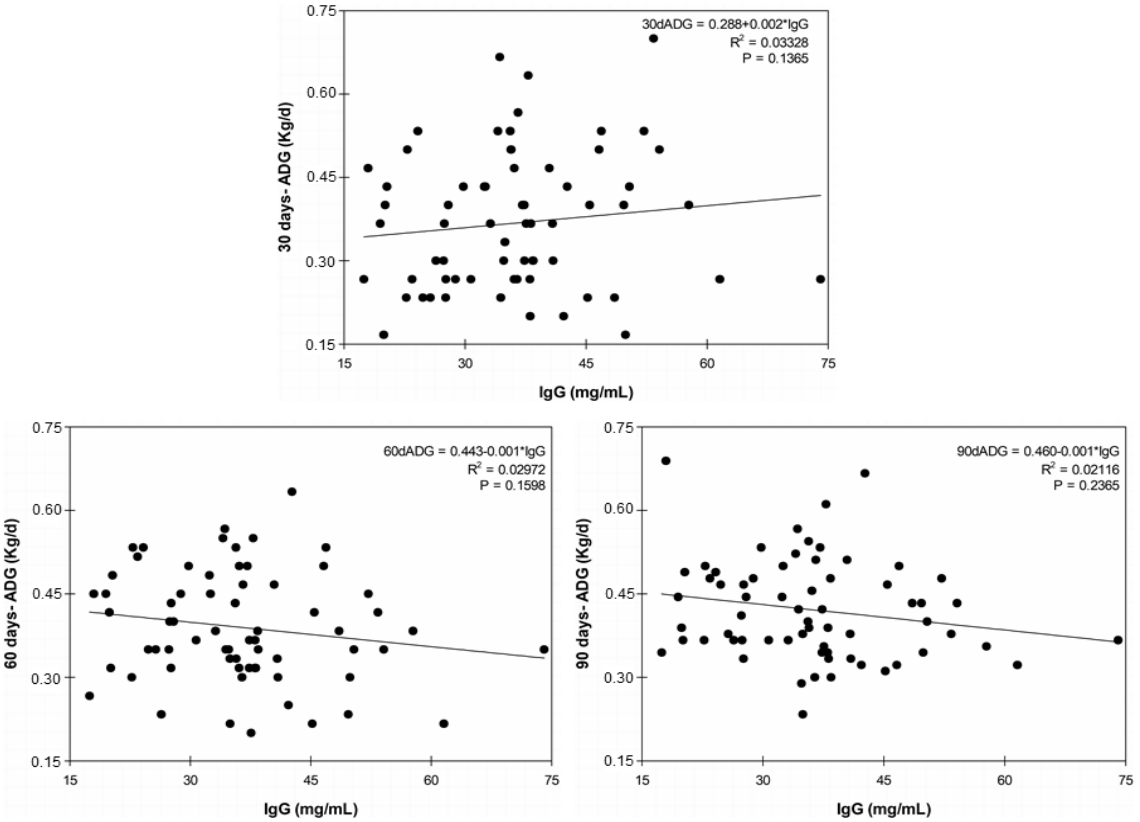

Figure 2. Scatter plots of average daily gain until 30, 60 and 90 days of age versus serum concentrations of immunoglobulin G (IgG) at 24h of buffalo calves that nurtured colostrum directly from the dams. The line represents the tendency of the data determined by simple linear regression.

Association between ADG and IgG at 24h were not detected in this study. Different from other studies where a positive correlation between serum IgG levels at 24-48h and ADG has been reported (Mastellone *et al.*, 2011; Elsohaby *et al.*, 2019). Nevertheless, it is important to thrive for higher levels of PIT, as it clearly enhances production efficiency on dairy farms, contrary to the statement that there is no benefit in surpassing the recommended IgG threshold for avoiding FPIT (Weaver *et al.*, 2000).

This absence of correlation between PIT evaluation parameters like total protein has been reported previously in a meta-analysis that did not find correlation between serum protein levels and growth performance in Holstein calves. The study found more relevant relationship of ADG with starter intake and the amount of milk replacer feed to the weaned calves (Bateman *et al*., 2012).

The biggest factor influencing growth performance is nutrition of both the calf, including there the weaning strategy and diet composition (Broadhead *et al.,* 2019). And the nutrition of the dam during pre-partum (Jolazadeh *et al*., 2019).

4. Conclusions

The hypothesis of the present study was denied, since there was no significant relation between serum concentrations of immunoglobulin G at 24h and weight or average daily gain of buffalo calves allowed to nurse the dam during the first 90 days of age.

Growth performance of buffalo calves seems to be much more related to dam's pre-partum nutrition and calf nutrition scheme. Nonetheless it is important to state proper growth is only achieved if calves are healthy, and this status is achieved by an efficient passive immunity transfer.

5. Acknowledgements

The authors acknowledge the contribution of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) in the scholarship granted - Finance Code 001.

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CHAPTER 4 –Serum biochemical profile of buffalo calves including acute phase proteins[3]

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ABSTRACT - Buffalos undergo various physiological changes after birth and on the first months of age, that affect the serum biochemical parameters used in clinical evaluation. The aim of this study was evaluate serum biochemical profile of healthy buffalo calves on the first three months of age. Blood samples from 146 Murrah buffalo calves, from both sexes, born from eutocic births, from clinically healthy buffalos, reared on a property in the State of São Paulo, were examined. Blood samples were taken on the first week of age at 24h – 48h (M1), 7-14 days (M2) 30-45 days (M3) and 60-90 days (M4) after birth for determination of gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatine kinase (CK), total protein, albumin, globulins, iron, total calcium, ionized calcium, magnesium, phosphorus, sodium, potassium, immunoglobulin G (IgG), immunoglobulin A (IgA), ceruloplasmin, transferrin, haptoglobin and α1-acid glycoprotein. The age of calves influenced the biochemical parameters. Observation of these variations are important because they allow for discrimination of physiological and pathological processes. The serum iron concentrations of buffalo calves were high, and therefore the supplementation of iron in these animals is not indicated.

Keywords: calf, colostrum, Murrah, neonate, newborn

RESUMO - Os búfalos sofrem várias alterações fisiológicas após o nascimento e nos primeiros meses de vida, que afetam os parâmetros bioquímicos séricos

[3] This chapter follows publications guidelines for the Journal Arquivo Brasileiro de Medicina Veterinária e Zootecnia – and will be send to publication.

utilizados na avaliação clínica. O objetivo deste estudo foi avaliar o perfil bioquímico sérico de bezerros saudáveis nos primeiros três meses de idade. Foram examinadas amostras de sangue de 146 bezerros Murrah, de ambos os sexos, nascidos de partos eutócicos, de búfalas saudáveis, criados em uma propriedade no Estado de São Paulo. As amostras de sangue foram coletadas na primeira semana de idade às 24h - 48h (M1), 7-14 dias (M2) 30-45 dias (M3) e 60-90 dias (M4) após o nascimento, para determinação da gamamaglutamiltransferase (GGT), fosfatase alcalina (ALP), aspartato aminotransferase (AST), creatina quinase (CK), proteína total, albumina, globulinas, ferro, cálcio total, cálcio ionizado, magnésio, fósforo, sódio, potássio, imunoglobulina G (IgG), imunoglobulina A (IgA), ceruloplasmina, transferrina, haptoglobina e α1-glicoproteína ácida. A idade dos bezerros influenciou os parâmetros bioquímicos. A observação dessas variações é importante porque permite a discriminação de processos fisiológicos e patológicos. As concentrações séricas de ferro nos bezerros de búfalo foram altas e, portanto, a suplementação de ferro nesses animais não é indicada.

Palavras-chave: bezerro, colostro, Murrah, neonato, recém-nascido

1. Introduction

Being originally from Asia water buffalos (*Bubalus bubalis*) are explored for meat and milk around the globe. With great adaptation they are raised both on cold areas as in Canada, to hot environments like Egypt. In Brazil the species has almost two centuries of development and is distributed from the tempered south to the hot and humid north.

Knowing the normal values of serum biochemical parameters is important in assessing organ and tissue damage in different conditions, as well as animal welfare. It allows for monitoring the metabolic condition of animal tissues, disorders in organ function and organism adaptation in nutritional and physiological changes (Kaneko *et al.*, 2008).

Factors such as species, race, age, rearing system, feeding, number of calving, among others, influence the results of serum biochemical components, and the identification of these factors allows a correct interpretation of the results (Klinkon and Jezek, 2012).

Despite the importance of the buffalo to world dairy production the available data around biochemical parameters to calves is still scarce. Therefore, the aim of this study was to establish a serum biochemical profile, including acute

phase proteins of buffalo calves from the first week of living until the third month of age.

2. Material and methods

The animals were raised in a semi-extensive property located in the State of São Paulo (23°34 S, 47°49 W), with annual milk production of 300,000 kg, with an average of 79 lactating animals and average individual production of 2,765 kg of milk in 300 days of lactation.

Only calves born from natural mating via eutocic births from clinically healthy dams that had suckled <4h after birth entered the study. Calves went through physical examination and considered clinically healthy when they did not present any alterations (Radostits *et al.*, 2000). After birth and natural colostrum intake, the umbilical cords of calves were treated with 2% iodine solution.

The calves used in this experiment (n=146; 78 male and 68 female) were free to suckle on their mothers during their first five days of age. After this, they were fed milk from a mammary quarter, not milked, until 90 days old. In addition, to the dairy diet, calves had access to *Brachiaria* spp. pastures and were fed with diet composed of soybean and maize.

All calves received treatment against endoparasites with albendazole sulfoxide 13.6% - (Ricobendazole 13,6%, Chemitec Ltda., São Paulo, Brazil) or albendazole 10% (Biozen, Biofarm, São Paulo Brazil) with seven and 21 days of age. Pulverization of fipronil 1% (Topline Red, Merial, Campinas, Brazil) was made if calves had a tick infestation, also an application of abamectin 1% (Abamec LA, Ourofino SA, Cravinhos, Brazil) or doramectin 1% (Dectomax, Zoetis, Campinas, Brazil) was made with two months of age. All animals were vaccinated against pasteurellosis (Tifopasteurina, Ceva, Paulínia, Brazil) and clostridiosis (Polistar, Vallé, Montes Claros, Brazil) with two months of age. All calves were characterized as negative for rotavirus on electrophoresis tests on polyacrylamide gel (PAGE).

Blood samples of 10 mL were collected by jugular venipuncture after local antisepsis at the following interval moments: at 24-48h (M1) after birth; at 7-14 (M2), 30-45 (M3) and 60-90 (M4) days of age. A vacuum collection system in

siliconized tubes without anticoagulant (Vacutainer, Bencton Dickinson, Franklin Lakes, USA) was used. Samples were clot at room temperature for 30 min and serum was collected after centrifugation at 2,000 x g for 10 minutes, and 2.0 mL aliquots of serum were separated and stored in micro tubes, previously identified and maintained at -20°C until laboratory tests.

Serum activities of gamma-glutamyltransferase (GGT) (modified Szasz method), aspartate aminotransferase (AST) (UV-IFCC kinetics method), creatine kinase (CK) (UV method) and alkaline phosphatase (ALP) (modified Bowers and McComb method) were determined, as well as serum concentrations of total protein (biuret method), albumin (bromocresol green method), total calcium (CPC method), magnesium (colorimetric – sulfonated Magon), phosphorus (modified Daly and Ertinghausen method) and iron (modified Goodwin method), using a set of commercial reagents (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil) were analyzed in a semiautomatic spectrophotometer (Labquest, Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil), using light of appropriate wavelength for each test.

Globulins were calculated from the arithmetic difference between total protein and albumin concentrations. Serum levels of ionized calcium, sodium and potassium were determined in an ion analyzer by the ion-selective electrode method (9180 Electrolyte Analyzer, Roche Diagnostics, Mannheim, Germany).

Protein fractionation of serum samples were determined using polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE), according to the technique proposed by Laemmli (1970). The concentrations of these proteins were determined by computerized densitometer (Shimadzu CS-9301 PC, Tokyo, Japan). As a reference, a marker solution with different molecular weights was used in addition to the purified bovine IgG protein (Sigma, St Louis, MO, USA).

Continuous variables were presented as the mean ± standard error of the mean (mean ± SEM). The comparison among variables for each interval of moment (M1, M2, M3 and M4) was performed by analysis of variance (ANOVA), using the GLIMMIX procedure of SAS® version 9.4 (SAS/STAT, SAS Institute

Inc., Cary, NC, USA) and the comparison between means was performed by the LSMeans test of the SAS software.

To determine the effect of the moment, the statistical models were formed by classificatory variables (P-values of Moment) and linear effect variables (Animal). The biochemical profile was measured by means of continuous response variables (AST; CK; GGT; ALP; TP; Albumin; Globulins; Ca; P; Mg; Fe; Na; K; iCa; IgG; IgA; Ceruloplasmin; Transferrin; Haptoglobin; α1-acid glycoprotein; dist = normal). The continuous response variables were subjected to the response scaling test through the Guided Data Analysis solution of SAS. Variables that did not follow these assumptions were transformed accordingly. Differences were considered significant when $P \leq 0.05$.

The research project was evaluated by the Ethical Committee on the Use of Animals (CEUA) of FCAV/UNESP, Jaboticabal campus, and approved under protocol 17.366/16.

3. Results and discussion

Serum activities of enzymes and serum concentrations of minerals and proteins of neonatal Murrah buffalo calves from birth to 90 days of age, are presented in Table. 1.

Aspartate aminotransferase (AST) and creatine kinase (CK) have both high activity on skeletal and cardiac muscle tissue, with AST also having hepatic origin. Their measuring is made together to evaluate tissue damage (Kaneko *et al*., 2008). There is an expected high activity on both enzymes on the first two days after birth (M1) as injuries to the skeletal muscle are due to the passage on the birth canal and calves begin to stand and exercise muscle tissue immediately after birth.

On the next two weeks (M2) and on the first two months (M3) AST levels decreases and on the third month of age (M4) return to similar levels as to the first days after birth. CK activities diminish at M2 and assume tendency of increase until M4. In this period calf growth is accelerated and buffaloes exhibit greater social interaction with the herd, running, jumping, and nodding. Resulting in higher levels of muscle tissue related enzymes (Klinkon and Jezek, 2012).

Serum activities of gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP) were higher in the first days after birth than on the subsequent three moments (P>0.0001). This is expected as there is an absorption of GGT and ALP content from colostrum origin on the first feeding. These enzymes are broken by calf gut over the first weeks of age and those values become more related to calf's own sera (Klinkon and Jezek, 2012; Souza *et al.,* 2019).

Table 1. Mean ± standard deviation of aspartate aminotransferase (AST), creatine kinase (CK), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP) serum activities, and serum concentrations of total protein (TP), albumin, globulins, total calcium (Ca), phosphorus (P), magnesium (Mg), iron (Fe), sodium (Na), potassium (K), ionized calcium (iCa), immunoglobulin G (IgG), immunoglobulin A (IgA), ceruloplasmin, transferrin, haptoglobin, α1-acid glycoprotein of neonatal Murrah buffalo calves at 24h – 48h (M1), 7-14 days (M2) 30-45 days (M3) and 60-90 days (M4) after birth.

Parameter	**M1**	**M2**	**M3**	**M4**
AST (U/L)	97.8±20.1a	77.8±24.5b	76.5±27.1b	93.3±27.1a
CK (U/L)	205±109a	178±97.9b	212±80.5cd	235±104d
GGT (U/L)	1,024±919a	130±107b	28.2±24.9b	28.1±25.3b
ALP (U/L)	806±648a	150±75.2b	103±43.8b	159±75.3b
TP (g/dL)	8.48±1.47a	8.02±1.07b	7.02±0.75c	6.73±0.59d
Albumin (g/dL)	2.12±0.29a	2.50±0.32b	2.74±0.58c	3.04±0.69d
Globulins (g/dL)	6.35±1.58a	5.52±1.13b	4.28±0.84c	3.69±0.80d
Ca (mg/dL)*	9.96±1.10a	10.2±1.08b	9.74±1.02a	9.88±0.93a
P (mg/dL)	7.96±2.13a	9.38±1.58b	9.02±1.45b	9.31±1.28b
Mg (mg/dL)	2.12±0.51a	1.96±0.46b	2.27±0.47c	2.36±0.40c
Fe (µg/dL)	96.2±49.3a	128±90.9b	186±82.0c	158±59.8d
Na (mMol/L)	137±2.86a	135±3.66b	133±3.59c	134±2.09c
K (mMol/L)	4.71±0.46a	5.01±0.44bc	4.96±0.47b	5.07±0.52c
iCa (mMol/L)	0.86±0.24a	0.81±0.22b	0.66±0.22c	0.53±0.08d
IgG (mg/dL)	3,407±1178a	2,425±816b	1,322±470c	1,029±636d
IgA (mg/dL)	542±130a	588±154b	557±159ab	492±147c
Ceruloplasmin (mg/dL)	77.0±33.2a	101±52.7b	167±52.8c	175±54.3c
Transferrin (mg/dL)	491±144a	614±165b	507±121a	541±97.7c
Haptoglobin(mg/dL)	8.19±4.13a	14.1±8.71bc	14.3±8.71b	12.1±11.1c
α1-acid glycoprotein (mg/dL)	5.41±4.76a	61.2±60.6b	98.3±77.4c	22.6±22.3d

Mean values followed by the same lower case letters on the same line do not differ significantly according to LSMeans test (P<0.0001).*P=0.0006

GGT activities at the first two weeks (M1) of age are still diminishing and as the calf grows older serum levels are more feasibly related to bile duct damage, being used to access the hepatic function together with AST on ruminants. GGT results at M3 and M4 were similar to those reported on Murrah buffalo calves of three months of age (28.6±3.2 U/L) on India (Khan *et al*., 2018).

Mean serum activities of ALP did not had significant changes (P>0.0001) after the first days of age (M1) until the third month of age (M3). The values on our study are within the reference interval of buffalo heifers and adult cattle (Kaneko *et al.* 2008; Ellah *et al*., 2014)

Serum concentrations of total protein (TP), albumin and globulins were all different (P>0.0001) between moments throughout the study. Together with globulins, albumin form serum TP content. As the calf ages albumin/globulin ratio of TP content changes: 0.33 (M1), 0.45 (M2), 0.64 (M3), 0.82 (M4). This is explained by the natural turnover of colostrum immunoglobulins acquired by passive immunity transfer (PIT) and the onset of calf's own immune response by synthetizing new immunoglobulins (Kaneko *et al.* 2008).

Mean serum concentrations of TP on the first three months (7.56±0.05 g/dL) on studied calves are higher than those reported on Murrah calves previously (6.15±0.50 g/dL), that difference is probably due to globulin concentrations being higher on the present study (Khan *et al*., 2018).

Albumin serum concentrations rise is occasioned by changes in hepatic tissue maturation and a diet composed more by forage and less by milk (Kaneko *et al*., 2008). Mean levels of albumin our study are above the levels of buffalo calves and below those from bovine calves (Klinkon and Jezek, 2012; Khan *et al*., 2018)

Calcium (Ca) serum concentrations presented a significant difference (P=0.0006) only in the sampling at 7-14 days (M2). With no biological impact as throughout the study mean levels of Ca remained within the reference interval of adult cattle (9.7-12.4 mg/dL). Ca is important in intracellular homeostasis for coagulation activation, and muscle contraction, is stored in bone tissue and is down regulated by phosphorus (P) content.

Means serum concentrations of P were significantly different (P>0.0001) only in the first days of age (M1). After that P values remained statistically unchanged. Our results are above the reference interval of adult cattle (5.60 – 6.50 mg/dL) and of buffalo calves on the first month, which may be due to the growth hormone stimulation resulting in greater renal absorption of phosphate (Kaneko *et al*., 2008; Souza *et al*., 2019). Similar high concentrations were reported on bovine calves previously (Klinkon and Jezek, 2012).

Magnesium (Mg) concentrations are affected by diet composition, in our study we observed an elevation of Mg levels after one month of age, were calves are feeding more of forage. Mean serum concentrations of magnesium (Mg) were within the reported reference interval of healthy buffalo heifers and adult cows (Kaneko *et al.* 2008; Ellah *et al*., 2014).

Iron (Fe) is of great importance to hemoglobin synthesis, hematopoiesis, immune response activation, and pathogen-host interactions (Kaneko *et al.*, 2008). Also iron is required to produce type 1 insulin-like growth factor (IGF-1), that is directly related to weight gain and growth performance (Prodanovic *et al*., 2014). Buffalo calves had different serum concentrations of iron during all the period studied (P>0.0001). With higher serum concentrations at M3, above the reference interval of adult cattle (57-162 µg/dL).

The serum concentrations of sodium, potassium, and ionized calcium had slight variation over the study period and were all within range of reference intervals of adult cattle. Electrolyte dosing is of great importance in assessment of animal health, since electrolyte imbalances lead to changes in the pH of body fluids, blood volume, heart rate, muscle contractions, and stability of cell membranes; it is also essential for the correction of acid-base imbalances and for cation-anion diet formulation (Kaneko *et al.*, 2008).

Immunoglobulin G (IgG) represents 86% of the total Igs on buffalo colostrum, completed by immunoglobulin A (IgA) and immunoglobulin M (IgM) 8% and 6% respectively (Dang *et al.*, 2009). The IgG serum concentration in buffalo calves increases rapidly after feeding colostrum, peaks between 1 and 3 days of age, and then decreases. Samples should be collected between 2 and 7

days old to provide the most accurate indication of passive transfer (Elizondo-Salazar and Heinrichs, 2009; Souza *et al.*, 2019)

Serum concentrations of immunoglobulin G (IgG) decreased significantly (P>0.0001) between moments. Calves had high IgG content in the first two days of age (M1), above the recommended to dairy calves (2,000-2,500 mg/dL). (Chigerwe *et al*., 2015). IgG absorbed via colostrum tend to last until the first month of age, when calf initiates his own immunological response (Tizard, 2009). And that is seen between the second (M3) and third month (M4) were there is less decrease in serum levels of IgG.

Calves had mean serum concentrations of immunoglobulin A (IgA) along the study above those related in dairy calves in the first month of age (Santos *et al*., 2013). Serum levels of IgA remained similar to those soon after birth until two months of age, then having a slight decrease. IgA is most commonly found in mucous secretions and acts by preventing infections through agglutination of microorganisms, binding to the intestinal wall and preventing the action of pathogens (Tizard, 2009).

Acute phase proteins (APPs) are important health biomarkers to access damage and inflammation on tissues, in buffalos it has been utilized to evaluate diarrhoea, respiratory related diseases and parasite infestation (El-Deeb and Iacob, 2012; Youssef *et al*., 2015; Clemente *et al*., 2016). APPs serve as a tool to diagnose detect and monitor diseases (Ulutas *et al.*, 2011).

Ceruloplasmin levels were minimal in the first days (M1) and first month (M2) of age. Between the second (M3) and third month of age (M4) no significant change (P>0.0001) was determined. This APP is related to iron metabolism, with also oxidase activity, and aids in diminishing iron availability to bacteria (Kaneko *et al*., 2008).

Serum concentrations of ceruloplasmin of this study were above those reported in healthy buffalo calves (El-Deeb and Iacob, 2012; Clemente *et al*., 2016). This may be due to our higher concentrations of Iron which are directly related to ceruloplasmin levels. That is one of the reasons to stablish a biochemical profile more related to each environmental characteristics.

Transferrin is a negative APP that binds to iron, limiting it availability to microorganisms and decreasing the growth and multiplication capacity of bacteria. Mean serum concentrations of transferrin fluctuated throughout the moments of the study, having higher means than the reported on healthy buffalo calves (Clemente *et al*., 2016; Santana *et al*., 2018).

Haptoglobin is the most utilized APP to access damage and disease in ruminants. It has great sensitivity and is increased within 24-48h after disease episode (Clemente *et al*., 2016; Santana *et al*., 2018). Calves on the present study had little variation in haptoglobin content between moments, with mean serum levels below those of healthy buffalos on other studies (El-Deeb and Iacob, 2012; Santana *et al*., 2018).

α1-acid glycoprotein has binds to various endogenous metabolites acting against infections, modulating immune reaction through neutrophil activation, inhibition of phagocytosis and platelet aggregation (Kaneko *et al.*, 2008). This APP had different serum concentrations between all studied moments (P>0.0001), with higher mean values in the first and second month of age, that were above of the means related in healthy adult buffaloes previously (El-Deeb and Iacob, 2012).

4. Conclusions

The age of the calves influenced the biochemical parameters Observation of these variations are important because they allow for discrimination of physiological and pathological processes. The serum iron concentrations of buffalo calves were high, and therefore the supplementation of iron in these animals is not indicated.

5. Acknowledgements

The authors acknowledge the contribution of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) in the scholarship granted - Finance Code 001. And also the Paineras do Ingaí farm in which the study was developed.

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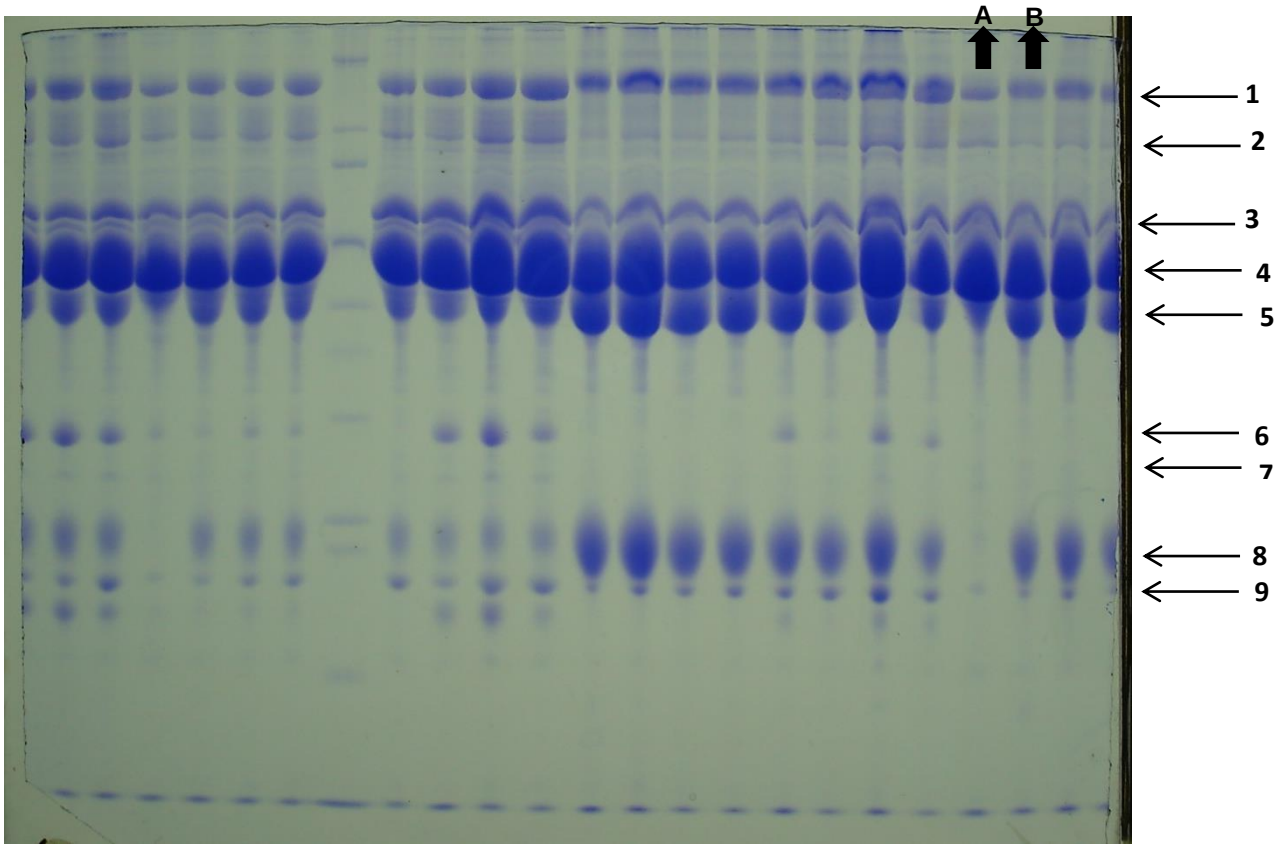
CHAPTER 5 – Final considerations

Evaluation of passive immunity transfer on buffalo calves by the determination of serum constituents like immunoglobulin G, gamma-glutamyltransferase and total protein on both calf's sera and colostrum of dams is important for the development of management strategies to reduce mortality rates on the neonatal phase.

With this information both farmer and veterinarian are able to choose dams with richer colostrum and form a proper colostrum bank. Also, knowing the expected values of healthy buffalo calves allows a better decision on weaning strategy. Thriving for better production outcomes throughout life, giving more profit to the farm by both selling heavier males and by providing better females for herd reposition.

APPENDIX

APPENDIX A - Example of SDS-PAGE fractioning and tracing.



1: IgA (167.000 Da)

2: Ceruloplasmin (113.000 Da)

3: Transferrin (78.000 Da)

4: Albumin (61.000 Da)

5: Heavy chain IgG (53.000 Da)

6: Haptoglobin (37.000 Da)

7: α1-acid glycoprotein (35.000 Da)

8: Light chain IgG (25.000 Da)

9: NIP (23.000 Da)

Figure 1A. Example of electrophoretic fractionation of blood serum samples from healthy Murrah buffalo calves, obtained by SDS-PAGE. A: blood serum sample before colostrum intake. B: blood serum sample 24 hours after birth. NIP: Non identified protein.

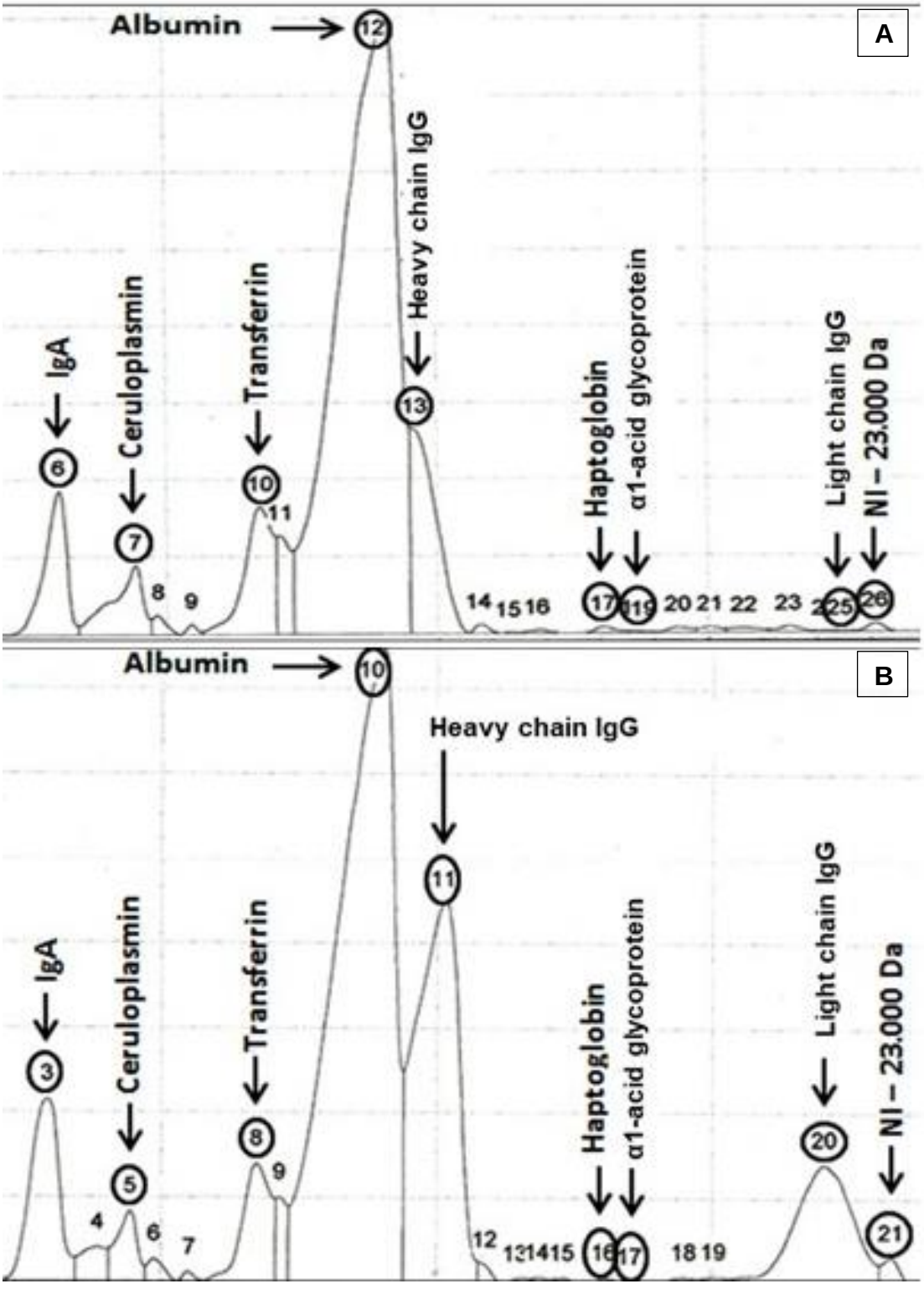


Figure 2A. Electrophoretic tracing of SDS-PAGE serum proteinogram of a healthy Murrah buffalo calf. A: blood serum sample before colostrum intake. B: blood serum sample 24 hours after birth. NI: protein not nominally identified.