PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

LÍVIA MAGOSSO RAMIRES

AVALIAÇÃO DO RDW EM EQUINOS DA RAÇA QUARTO DE MILHA SAUDÁVEIS E HOSPITALIZADOS, E COMO BIOMARCADOR DA INFECÇÃO POR *Strongylus vulgaris*

Presidente Prudente – SP
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Tese apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutor em Fisiopatologia e Saúde Animal.

Área de Concentração: Fisiopatologia Animal

Orientador: Prof. Dr. Vamilton Alvares Santarém

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Área de Concentração: Fisiopatologia e Saúde Animal.

Presidente Prudente, 20 de novembro de 2018.

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"Aprendamos com a Natureza e adotemos a brandura por diretriz de nossas realizações para a vida mais alta, mas não a brandura que se acomoda na inércia, com a perturbação e com o mal e sim aquela que se baseia na paciência construtiva, que trabalha incessantemente e persiste no melhor a fazer, ultrapassando os obstáculos que a ignorância lhe atira à estrada e superando os percalços da luta, a sustentar-se no serviço que não esmorece e na esperança fiel que confia, sem desânimo, na vitória final do bem."

(Chico Xavier)

RESUMO

Avaliação do RDW em equinos da raça Quarto de Milha saudáveis e hospitalizados, e como biomarcador da infecção por *Strongylus vulgaris*

RDW (Amplitude de Distribuição das Células Vermelhas do Sangue) é um índice hematimétrico usado para diagnosticar anisocitose. Na medicina humana, o RDW tem sido amplamente utilizado como um preditor e biomarcador de várias doenças, como a isquemia aguda mesentérica. Em equinos, esta isquemia tem sido associada à cólica verminótica, uma síndrome potencialmente fatal desencadeada pelas larvas do nematódeos *Strongylus vulgaris*. No entanto, existem poucos estudos na medicina equina sobre o RDW e sua relação com a infecção por *S. vulgaris,* bem como a padronização do RDW-CV nesta espécie. Na primeira fase, o objetivo foi o de comparar os valores de RDW-CV em equinos da raça Quarto de Milha hígidos e em equinos atendidos em um Hospital Veterinário Escola. Um total de 176 equinos foram avaliados, 95 dos quais eram hígidos, criados em 27 fazendas da região de Presidente Prudente, São Paulo, e 81 equinos hospitalizados. Amostras de sangue foram avaliadas para obtenção do índice RDW, a partir de contador automatizado de células. Os valores de RDW em equinos hospitalizados (18,6%) foram significativamente maiores ($p = 0,0006$) que os observados em equinos saudáveis (17,8%). O valor de RDW-CV (17,8%) pode ser usado como referência para equinos da raça Quarto de Milha. E sugere-se que mais estudos sejam necessários para avaliar o uso de RDW como biomarcador ou preditor de várias outras doenças em equinos. Para a segunda fase do estudo, foi hipotetizado que o RDW-CV poderia também ser usado como um biomarcador para infecção por *Strongylus vulgaris*. Foram avaliados 160 equinos sadios (Quarto de Milha e mestiço de Quarto de Milha), com pelo menos dois anos de idade e de ambos os sexos. O ELISA apresentou resultado positivo em 80% das amostras, no entanto, a coprocultura e a PCR específica foram negativas para *S. vulgaris*, apesar do elevado número de animais (91,25%) positivos para contagem de ovos (> 50 epg). Nenhuma evidência de associação foi observada entre RDW-CV e teste ELISA. Estes resultados são discutidos e, apesar da falta de associação, mais investigações são necessárias para avaliar o papel do RDW como biomarcador/preditor de arterite verminótica em equinos apresentando cólica.

Palavras-chave: cavalos. Cólica. Diagnóstico. ELISA. Marcador inflamatório. Parasito. PCR

ABSTRACT

Evaluation of RDW in healthy and hospitalized Quarter Horses, and as a biomarker of infection by *Strongylus vulgaris*

RDW (Red Blood Cell Distribution Width) is a hematimetric index used to diagnose anisocytosis. In human medicine, RDW has been widely used as a predictor and biomarker of various diseases, such as acute mesenteric ischemia. In equines, this ischemia has been associated with vermintic colic, a potentially fatal syndrome triggered by the larvae of the nematodes *Strongylus vulgaris*. However, there are few studies in equine medicine on RDW and its relation to *S. vulgaris* infection, as well as the standardization of RDW-CV in this species. In the first phase, the objective was to compare the values of RDW-CV in equine horses of the healthy Quarter Horses and in horses attended in a Veterinary Hospital School. A total of 176 horses were evaluated, 95 of which were healthy, reared on 27 farms in the region of Presidente Prudente, São Paulo, and 81 horses hospitalized. Blood samples were evaluated to obtain the RDW index, from automated cell counter. The RDW values in hospitalized horses (18.6%) were significantly higher ($p = 0.0006$) than those observed in healthy horses (17.8%). The RDW-CV value (17,8%) can be used as a reference for horses of Quarter Horse breed. It is suggested that further studies are needed to evaluate the use of RDW as a biomarker or predictor of several other diseases in horses. For the second phase of the study, it was hypothesized that RDW-CV could also be used as a biomarker for *Strongylus vulgaris* infection. We evaluated 160 healthy horses (Quarter Horse and Quarter Horse mixed), at least two years old and of both sexes. The ELISA showed a positive result in 80% of the samples; however, the co-culture and the specific PCR were negative for *S. vulgaris*, despite the high number of animals (91.25%) positive for egg counts (> 50 epg). No evidence of association was observed between RDW-CV and ELISA. These results are discussed and, despite the lack of association, further investigations are needed to evaluate the role of RDW as a biomarker/predictor of vermintic arteritis in horses with colic.

Key-words: Horses. Colic. Diagnosis. ELISA. Inlammatory marker. Parasite. PCR

LISTA DE ABREVIATURAS

AVC - Acidente Vascular Cerebral

°C- Grau Celsius

DNA - Ácido Desoxirribonucleico

DPOC - Doença Pulmonar Obstrutiva Crônica

EDTA - Ácido Etilenodiaminotetracético

ELISA - Enzyme Linked Immunonosorbent Assay

EUA - Estados Unidos da América

IC - Intervalo de Confiança

L3/L4 - Larvas de estágio 3 e 4

MCV - Volume Corpuscular Médio

mL - Mililitro

min - Minutos

OPG - Ovos por Grama de Fezes

OR - *Odds Ratio* (razão de chance)

p ou p value – valor de p

Pb - Pares de bases

PCR - Reação em Cadeia da Polimerase

% - Porcentagem

QM - Quarto de Milha

RDW - Amplitude de Distribuição dos Eritrócitos

RDW-CV - Amplitude de Distribuição dos Eritrócitos medido como Coeficiente de Variação

RM - Raça Mestiça

s – Segundos

TM – Trade Mark (marca registrada)

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1 INTRODUÇÃO E REVISÃO DE LITERATURA

Na rotina da Patologia Clínica, o uso do Volume Corpuscular Médio (MCV) e a contagem de reticulócitos são os dois principais critérios tradicionais para a classificação inicial de distúrbios anêmicos (ALBAYRAK et al., 2014). Nos últimos anos os contadores eletrônicos hematológicos têm sido apontados como mais eficazes e práticos na análise da série vermelha sanguínea, com o fornecimento de parâmetros mais objetivos para avaliação da anemia e eritropoiese, como o RDW (Red Blood Cell Distribution Width) (ALIS et al., 2016). Este índice, por meio de uma análise quantitativa, reflete o grau de heterogeneidade entre as hemácias, representando o coeficiente de anisocitose eritrocitária (ANDRIICHUK et al., 2017).

Em estudos recentes, pesquisadores têm mostrado que o RDW pode ser um importante marcador para processos inflamatórios de caráter crônico em seres humanos, e como preditor de várias enfermidades (BREDA et al., 2015; TANG; KATZ, 2016; ANDRIICHUK et al., 2017), como diversos tipos de neoplasias (SERETIS et al., 2013; ALBAYRAK et al., 2014; WANG et al., 2014; HUANG et al., 2016), doenças mieloproliferativas crônicas (HACIBEKIROGLU et al., 2015), DPOC (doença obstrutiva pulmonar crônica) (KOMA et al., 2013), aneurisma (RIEDL et al., 2014) e cardiopatias (BREDA et al., 2015; DUCHNOWSKI et al., 2015; ELLINGSEN et al., 2018). Pacientes com sintomas de AVC (Acidente Vascular Cerebral), em menos de 24 horas da injúrias, apresentaram RDW significativamente mais elevados que aqueles que sofreram danos mais leves (KARA et al., 2015). Esses autores concluíram que o RDW foi um preditor da gravidade do AVC.

A obtenção do RDW é simples, acessível e de baixo custo (BALARIN et al., 2006), o que o torna uma ferramenta simples e com múltiplas aplicações clínicas (DUCHNOWSKi et al., 2015).

Apesar do crescente interesse nesta ferramenta de diagnóstico na medicina humana (ELLINGSEN et al., 2018), estudos sobre RDW em animais ainda são escassos.

Na clínica de equídeos o RDW tem sido empregado para avaliar a relação da variação do tamanho das hemácias com a intensidade de exercícios praticados pelos animais (HOLANDA et al., 2013; MELO et al., 2013) ou com certos tipos de dietas (MÉLO et al., 2012; MÉLO et al., 2016). Porém, não há trabalhos avaliando a

relação do RDW com o estado clínico dos equinos, bem como a padronização de seus valores para determinadas raças, como Quarto de Milha (QM).

Os equinos são animais de grande importância econômica, que podem ser utilizados em esporte, lazer e trabalho. Só no Brasil, o plantel contabiliza algo em torno de 5.600.000 animais, ocupando a quarta maior criação no mundo (BRASIL, 2015).

O uso do cavalo nas práticas esportivas, como os da raça Quarto de Milha, é o segmento mais lucrativo da equideocultura nacional, com a participação estimada de 50 mil cavalos atletas (ANUALPEC, 2012). Além da prática esportiva, os cavalos são utilizados em atividades de trabalho que ajudam no desenvolvimento da agropecuária nacional (ROSA, 2014). Desta forma, a prevenção e o diagnóstico precoce das patologias que mais comumente acometem esta espécie são de extrema importância para manutenção da higidez dos animais (FINGER et al., 2013).

Dentre as patologias de maior ocorrência no cenário equestre, está a síndrome do abdômen agudo, popularmente chamada de “cólica equina”, que constitui a principal causa de óbito em animais adultos, o que acarreta prejuízos econômicos devido aos elevados custos com o tratamento (CANELLO, 2013; ROSA, 2014).

Nos equinos, a síndrome pode estar correlacionada com vários fatores, que vão desde uma produção excessiva de gases no estômago, fermentação de alimentos, obstruções, torções intestinais até infecções parasitárias (NIELSEN et al., 2015). Isso se deve ao fato de que os equinos são um dos animais mais susceptíveis aos parasitos gastrintestinais, podendo abrigar várias espécies de helmintos concomitantemente (NIELSEN et al., 2016), sendo os nematódeos os mais frequentes (MORALES et al., 2012; FABIANI; LYONS; NIELSEN, 2016).

Em geral, os cavalos hospedam alguns gêneros de endoparasitos em diferentes graus de infecção e, embora muitos animais parasitados não se apresentam clinicamente doentes, alguns podem desenvolver patologias importantes, como arterite tromboembólica mesentérica cranial, comprometimento da circulação intestinal e cólica verminótica (ANDRADE; SOBRAL; SILVA, 2009; NIELSEN et al., 2015).

Dentre os endoparasitas que infectam os equinos, *Strongylus vulgaris* é considerado um dos mais prevalente e patogênico em todo o mundo (ANDERSEN et al., 2013). No Brasil, já na década de 30, foi destacada a importância do estudo da

estrongilose dos equídeos, para conhecimento sobre a predominância das espécies do gênero Strongylus, e assim, informações acerca do diagnóstico e etiologia dos aneurismas helmínticos causados por *S. vulgaris* (PINTO; PROENÇA, 1938).

Os infartos intestinais do tipo não estrangulativos, resultam de obstrução vascular e, nos equinos, sua causa é o tromboembolismo nas artérias mesentéricas devido à infecção por *S. vulgaris* (VEIGA et al., 2011).

A patogenia da infecção por *S. vulgaris* é ocasionada pelas suas larvas durante a migração endoarterial, onde provocam hemorragias na mucosa do íleo, ceco e cólon, edema da submucosa e dilatação de pequenas artérias, veias e capilares (ALMEIDA; MELO, 2010). Na submucosa, ocorre a muda de L3 para L4, quando as larvas penetram em arteríolas e migram pelo sistema arterial até alcançar as artérias cecais (lateral e medial) e o ramo cólico da artéria ileocólica, promovendo o depósito de fibrina na camada íntima arterial e a formação de lesões tromboembólicas (aneurisma) que comprometem a circulação do órgão e ocasionam isquemia local ou generalizada das alças intestinais, caracterizando a cólica por arterite verminótica (REICHMANN et al., 2001).

Aproximadamente duas a três semanas pós-infecção, pode ocorrer infarto do íleo, ceco e cólon, associado à trombose e espessamento da artéria mesentérica cranial e seus ramos, culminando nos sinais clínicos de cólica isquêmica, que pode ser fatal para o equino acometido (NIELSEN et al., 2012; PILO et al., 2012; BORJI et al., 2014; NIELSEN et al., 2015; NILSEN et al., 2016).

A infecção por *S. vulgaris* pode apresentar um período de infecção subclínica, com sinais não característicos apenas da cólica verminótica, como: desconforto abdominal, letargia e/ou decúbito, anorexia, diminuição de ganho de peso e anemia (ANDRADE et al., 2009; MORALES et al., 2011; BOTELHO et al., 2012; CARVALHO, 2016). Mas também pode apresentar um silencioso processo de isquemia mesentérica com a rápida evolução para o óbito do animal (THOMASSIAN, 2005).

Para o diagnóstico da infecção por *S. vulgaris* em equinos, o exame coproparasitológico tem sido usado como o de eleição. Entretanto, este diagnóstico pode resultar em falso-negativo para tal nematódeo em decorrência da presença de larvas imaturas (REINEMEYER; NIELSEN, 2013). Dessa forma, mesmo que haja infecção, o tratamento pode ser incorreto, o que pode levar o animal a óbito muito rapidamente pela cólica verminótica.

Lhamas et al. (2015) citam que a relação existente entre altas infecções parasitárias e alterações hematológicas tem sido, cada vez mais, objeto de estudos. Assim, novas ferramentas de diagnóstico, como o RDW, poderiam detectar processos inflamatórios do aparelho gastrintestinal ainda na fase subclínica, e proporcionar o tratamento mais preciso, que aumentaria as chances de sobrevida do animal.

Diante dessas premissas, foram desenhados dois estudos para avaliar os objetivos apresentados:

- Comparar os valores do índice RDW-CV para equinos saudáveis da raça Quarto de Milha;

- Investigar a utilização do RDW-CV como um biomarcador para infecção de equinos por *Strongylus vulgaris*.

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2 ARTIGO CIENTÍFICO I*

Red Blood Cell distribuiton Width (RDW-CV) em cavalos Quarto de Milhas: comparação entre animais saudáveis e hospitalizados.

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Resumo
RDW (Amplitude de Distribuição das Células Vermelhas do Sangue) é um índice hematimétrico usado para diagnosticar anisocitose. Na medicina humana, o RDW tem sido amplamente utilizado como um preditor e biomarcador de várias doenças. No entanto, existem poucos estudos sobre este marcador na medicina equina. Este estudo teve como objetivo comparar os valores de RDW-CV em eqüinos saudáveis e hospitalizados da raça Quarto de Milha. Um total de 176 animais foram avaliados, 95 dos quais eram saudáveis, criados em 27 fazendas, e 81 tratados em um Hospital Veterinário. Amostras de sangue foram avaliadas usando um analisador hematológico automatizado para obter RDW. Os valores de RDW em eqüinos hospitalizados (18,6%) foram significativamente maiores (p = 0,0006) que os observados em equinos saudáveis (17,8%). Os valores de RDW apresentados podem ser usados como referência para equinos da raça Quarto de Milha.
Palavras-chaves: equino, hematologia, valores de referência, biomarcador

Red Blood Cell Distribution Width (RDW-CV) in Quarter Horses: A comparison between healthy and hospitalized animals

Abstract

RDW (Red blood cell distribution width) is a hematimetric index used to diagnose anisocytosis. In human medicine, it has been widely used as a predictor and biomarker of various diseases, but there are few comparable studies in equine medicine. We aimed to compare RDW values between healthy and hospitalized Quarter Horses (QH). A total of 176 QH were evaluated, 95 healthy animals reared on 27 farms, and 81 animals treated at a Veterinary School Hospital. Blood samples were evaluated using an automated hematological analyzer to obtain RDW. RDW values in hospitalized horses (18.6%) were significantly higher than in healthy ones (17.8%, p = 0.0006). Values from our healthy group can be used as a reference for QH. We suggest that further studies are needed to evaluate the use of RDW as a biomarker or predictor of various disorders or diseases in equines.
Keywords: equine; RDW; hematology; biomarker

Introdução

Na rotina da patologia clínica o uso do Volume Corpuscular Médio (MCV) e a contagem de reticulócitos são os dois principais critérios tradicionais para a classificação inicial de distúrbios anêmicos (Bessman et al. 1983). Nos últimos anos, o uso de contadores hematológicos automáticos propiciou diagnósticos mais precisos e rapidez na análise, além de gerar novas medidas eritrocitárias de garnde importância para a avaliação da eritropoiese (Matos et al. 2008), como o RDW-CV (Red Blood Cell Distribution Width), que, por meio de uma análise quantitativa, reflete o grau de heterogeneidade entre as hemácias, representando o

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coeficiente de anisocitose eritrocitária (Riedl et al. 2014; Wang et al. 2014; Ellingsen et al. 2018). A obtenção do RDW é simples, acessível e de baixo custo (Kisaoglu et al. 2014), o que o torna uma ferramenta simples e com múltiplas aplicações clínicas (Salvagno et al. 2015).

Em estudos recentes, pesquisadores têm mostrado que o RDW-CV (que será citado no texto como RDW) pode ser um marcador para processos inflamatórios de caráter crônico em seres humanos, e como preditor de várias enfermidades (Tang and Katz 2006; Kisaoglu et al. 2014; Riedl et al. 2014; Özer et al. 2015). Pacientes com sintomas de AVC (acidente vascular cerebral), em menos de 24 horas das injúrias, apresentaram RDW significativamente mais elevados que aqueles que sofreram danos mais leves. Esses dados mostraram a utilidade do RDW para predizer a gravidade e os resultados funcionais do AVC (KARA et al., 2015).

Na clínica de equídeos o RDW tem sido empregado para avaliar a relação da variação do tamanho das hemácias com a intensidade de exercícios praticados pelos animais (Balarin et al. 2006; Miranda et al. 2011) ou com certos tipos de dietas (Mélo et al. 2012; Mélo et al. 2016). Porém, não há trabalhos avaliando a relação do RDW com o estado clínico dos equinos, bem como a padronização de seus valores para determinadas raças, como Quarto de Milha (QM).

Dada a importância que o RDW tem se mostrado em estudos como preditor de processos inflamatórios e de outras injúrias na medicina humana, o presente estudo teve por objetivo a comparação dos valores do RDW-CV em equinos da raça QM sadios e atendidos em um Hospital Veterinário Escola.

Material e Métodos

O estudo foi aprovado pelo Comitê de Ética em Uso de Animais (CEUA) da Instituição (oculto para revisão), e realizado no período de março de 2016 a junho de 2017.

Foram avaliadas amostras de sangue de 176 equinos da raça Quarto-de-Milha (95 equinos sadios e 81 hospitalizados).

Os animais sadios foram selecionados, independentemente do sexo, idade e tipo de manejo, em 14 propriedades visitadas na região do estudo, e a higidez foi avaliada através de exame clínico segundo o protocolo descrito por Blood et al. (2000). Em seguida, foram coletadas amostras de sangue (10 ml) por punção venojugular, em tubo a vácuo com anticoagulante EDTA (ácido etileno-diaminotetracético-potássico) a 10% para realização do hemograma. As coletas ocorreram sempre no período da manhã, com os animais em repouso, antes de suas atividades diárias.

Em relação aos animais hospitalizados, foi realizado um levantamento dos valores hematológicos dos equinos atendidos na rotina do Hospital Veterinário Escola no período do estudo.

As amostras de sangue foram processadas em analisador automático (pocH-100iv-DIFF, Sysmex Roche, Japão), para obtenção dos valores hematológicos, incluindo a amplitude de distribuição do tamanho do eritrócito (RDW-CV).

Os valores de RDW-CV foram comparados entre animais hospitalizados e sadios, e foram avaliadas as influências da raça, do sexo e idade dos animais.

O programa GraphPad Prism (versão 7.04, GraphPad Software Inc., La Jolla, EUA) foi usado para análises estatísticas. Os valores de RDW foram submetidos ao teste de normalidade. Os dados não-paramétricos foram analisados pelo teste de Mann-Whitney e os paramétricos pelo teste t, para avaliação da influência do sexo ou raça. ANOVA foi empregada para avaliação da influência da idade sobre o RDW. A correlação entre RDW e

outros parâmetros hematológicos (Hematócrito e VCM) foi obtida pela correlação de Pearson. O qui-quadrado foi empregado para avaliar a associação entre tipo de manejo e atividade dos equinos com elevação de RDW. Foram considerados significativos os valores com $p<0,05$.

Resultados

No presente estudo foram incluídos 81 equinos hospitalizados da raça QM, representando 71,7% dos equinos atendidos no HV (n=113) no período de 2016 a 2017.

Foram admitidos para realização de castração sete equinos no grupo hospitalizados, e o RDW foi obtido um dia anterior a cirurgia. A média dos valores de RDW desses equinos foi a mesma observada nos outros equinos hospitalizados (RDW = 18.6%, variação 16.0 - 19.1).

Verificou-se diferença significativa (p=0,0006) entre os valores médios de RDW (Tabela 1) dos animais hospitalizados (18,6%) e sadios (17,8%), sendo que 65,4% e 46,3%, respectivamente, apresentaram valores acima da média dos sadios. Foi observado também que o valor de RDW nos animais hospitalizados foi 4,5% superior ao outro grupo estudado.

Foi também observada correlação negativa entre RDW e o valor do hematócrito ($p<0,0009$; r= -0,3634) e do VCM ($p<0,0091$; r= -0,2882) nos animais hospitalizados, fato não observado nos animais sadios. Houve uma associação (p= 0.0333; OR= 3.115; 95% IC= 1.133 - 8.563) entre a presença de anisocitose e valor de RDW acima dos considerados normais no presente estudo (17.7%).

Não houve influência do sexo dos equinos sobre os valores médios de RDW quando foram comparados os animais em seus respectivos grupos (hospitalizados e sadios). Entretanto, esses valores foram significativamente maiores nos equinos machos hospitalizados quando comparado aos machos hígidos, o que não ocorreu com as fêmeas (Tabela 2).

Em relação à idade, não houve influência dessa variável no RDW dos equinos sadios. Nos hospitalizados com idade inferior a dois anos, o RDW foi significativamente maior que nos com idade entre 2 a 10 anos. Quando os dois grupos foram comparados, a média nos hospitalizados com idade inferior a dois anos e de 2 a 10 anos foi significativamente maior que a dos em higidez (Tabela 3).

Com relação ao manejo, os valores do RDW dos equinos estabulados (17,8±0,8%) e daqueles mantidos a campo foram muito próximos (17,8±1,2%), seguindo a média observada na população de animais sadios (17,8±0,8%). Não houve diferença significativa quando foram comparadas as atividades dos animais (esporte, trabalho, lazer/estimação).

Discussão

O presente estudo mostrou que os valores de RDW em animais hospitalizados foram significativamente superiores aos dos animais hígidos.

Considerando-se os animais em higidez sadios, os valores observados no nosso estudo (RDW = 17,8%) foram idênticos aos observados em outro estudo com 29 equinos também sadios e da raça QM (RDW = 17,8%), antes da atividade física (Miranda et al., 2011). Os dados de Carvalho et al. (2016), também avaliando equinos QM (n= 22), foram 1,6 vezes maiores (28,4%) que os obtidos no nosso estudo. A discrepância desses resultados provavelmente se deu por causa dos diferentes modelos de analisador automático. O equipamento do nosso estudo e no conduzido por Miranda et al. (2011) foram do mesmo modelo, diferentemente daquele utilizado por

Carvalho et al. (2016). A performance do modelo de analisador usado no nosso estudo foi considerada excelente para avaliação de dados hematológicos de equinos (Riond et al., 2011).

A influência do analisador hematológico sobre a variação nos valores médios de RDW tem sido também descrita em cães (Souza et al., 2012). Segundo os autores, os equipamentos utilizam métodos diferentes que resultam em padrões de contagens distintas.

Em estudos com pacientes humanos, em decorrência da discrepância de resultados do RDW, foi postulado que a utilização de um mesmo modelo de analisador hematológico permite uma alta comparabilidade de dados (Alis et al., 2016), enquanto a falta de harmonização de resultados é uma séria limitação para o uso de valores de referência universais (Salvagno et al., 2015).

A raça é outra variável que deve ser considerada na avaliação do RDW de equinos (Holanda et al. 2013). Os nossos resultados foram inferiores aos observados em equinos da raça Holsteiner, de 36,1% (Andriichuk and Tkachenko 2017); Puro Sangue Inglês, entre 26,9% e 27,3% (Balarin et al. 2001; Balarin et al. 2006); e Mangalarga Marchador, entre 18,8% e 20,4% (Mélo et al. 2012; Holanda et al. 2013; Melo et al. 2013; Mélo et al. 2016). As avaliações hematológicas dos animais Mangalarga Marchador foram realizadas com o mesmo equipamento do presente estudo, enquanto os referentes aos equinos Holsteiner e PSI foram de modelos distintos. Dessa forma, é possível que, além da variável tipo de equipamento, o fator racial pode ser determinante nos valores de RDW.

De acordo com Holanda et al. (2013), as condições de manejo devem ser consideradas como fatores importantes na determinação dos intervalos de referência hematológica para a espécie equina. No nosso estudo, as condições de manejo (estabulados ou mantidos a campo) e a função do animal (trabalho, esporte ou lazer) não exerceram influência sobre os valores de RDW.

Outra proposta do nosso estudo foi comparar os valores de RDW entre animais hígidos e atendidos no hospital. Os valores do biomarcador nos animais hospitalizados foram significativamente superiores aos dos animais sadios, assim como o número de animais com RDW aumentado.

O RDW é um excelente parâmetro para classificação de anemias (Riedl et al. 2014; Ellingsen et al. 2018). Foi observada uma associação entre anisocitose e valor de RDW acima da média dos animais hígidos, e uma correlação negativa entre RDW e o valor do hematócrito e do VCM apenas nos animais hospitalizados. No estudo de Carvalho et al. (2016), não foram encontradas diferenças significativas quando correlacionado o VCM e RDW entre os grupos machos e fêmeas. Vale ressaltar que o estudo foi realizado com animais em atividade física, que tem forte influência no VCM, o que é também afirmado por Piccione et al. (2001). Em sua pesquisa, Balarin et al. (2006) justificaram tanto as oscilações em VCM quanto em RDW como decorrentes das diferentes raças e protocolos laboratoriais, ressaltando a importância do estabelecimento de valores de referência para cada região e espécie/raça.

O RDW tem sido descrito como preditor prognóstico de várias enfermidades em seres humanos, incluindo diversos tipos de neoplasias (Seretis et al. 2013; Wang et al. 2014; Albayrak et al. 2014; Huang et al. 2016), doenças mieloproliferativas crônicas (Hacibekiroglu et al. 2015), DPOC (doença obstrutiva pulmonar crônica) (Koma et al. 2013), aneurisma (Riedl et al. 2014) e cardiopatias (Breda et al. 2015; Duchnowski et al. 2015; Ellingsen et al. 2018). Riedl et al. (2014), por exemplo, observaram que para cada 1% de aumento do RDW houve 11% de aumento no risco de mortalidade tromboembolismo venoso em pacientes humanos. No nosso estudo, os valores médios nos equinos hospitalizados foi 4,5% acima da média dos animais sadios. Os

animais hospitalizados incluídos no nosso estudo apresentaram as mais diversas causas de hospitalização, com algum quadro de processo infeccioso/inflamatório, que poderiam ter influenciado o aumento de RDW.

O sexo e a idade dos animais foram também avaliados quanto aos valores de RDW. Balarin et al. (2001) postularam que os valores de referência de RDW podem ser usados independentemente do sexo dos animais, tese que pode ser corroborada pelos resultados observados em outros estudos com equinos (Miranda et al. 2011; Mélo et al. 2012; Holanda et al. 2013), e também por nosso estudo, uma vez que a variável sexo não influenciou no RDW quando os dois grupos estudados foram comparados separadamente.

No presente estudo, as médias de RDW não diferiram entre as faixas etárias quando da comparação entre animais sadios e hospitalizados. Em contrapartida, nos animais hospitalizados com idade inferior a dois anos, o RDW foi significativamente maior que nos animais com idade 2 a 10 anos. No estudo de Holanda et al. (2013), com Mangalarga Marchador, os valores de RDW foram significativamente maiores nos animais com idade entre 2 a 6 anos, em relação aos animais mais velhos. Os autores, porém, não avaliaram animais com menos de 2 anos de idade, não permitindo a comparação entre os resultados. Alis et al. (2016) verificaram que a idade pode ser considerada como importante fator de confundimento na interpretação de RDW em humanos, como observado no nosso estudo, uma vez que os animais jovens podem apresentar valores de RDW maiores que os considerados como de referência para a população geral.

Balarin et al. (2006) estudando equinos submetidos a exercícios de diferentes intensidades observaram um aumento significante dos valores de RDW. Outros estudos também relataram este aumento após exercício (Mélo et al. 2012; Mélo et al. 2016). Já no estudo de Miranda et al. (2011), os valores de RDW permaneceram inalterados depois da atividade física exercida pelos animais. Os pesquisadores atribuíram este resultado a provável liberação mínima de células pelo baço que foi insuficiente para provocar grande heterogeneidade entre as hemácias. No nosso estudo, os animais hospitalizados e os avaliados a campo estavam em repouso, o que indica que o aumento de RDW nos animais hospitalizado não foi decorrente de atividade física.

O uso do RDW como ferramenta na clínica equina deve ser considerado, uma vez que o mesmo pode representar um marcador de vários processos inflamatórios, com baixo custo e de fácil e rápida execução. Uma das principais limitações em estudos com equinos, e também com outras espécies de animais, é o número amostral. Os estudos com humanos são realizados com grande número de indivíduos que permitem uma análise retrospectiva ou prospectiva de informações, e o uso do RDW como indicador de morbidade ou preditor de várias enfermidades.

Os nossos resultados comparados com os de outros estudos permitem inferir que a variação dos valores de referências do RDW pode ocorrer entre diferentes raças e ser influenciada pelo equipamento utilizado para realização da análise hematológica.

Em conclusão, os nossos dados apresentam valores de RDW que podem ser usados como referência para equinos da raça Quarto de Milha.

Fabricantes

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Declaração de conflito de interesse

Os autores declaram não haver conflito de interesses.

Ética da pesquisa animal

Neste estudo foram utilizados cavalos com permissão por escrito dos proprietários.

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Autoria

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Diretrizes éticas

O artigo é original, não foi submetido ou publicado em outro lugar e tem a aprovação de todos os autores.

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Tabela 1. Valores médios de RDW-CV (%) em equinos da raça Quarto-de-Milha (QM), hospitalizados (n= 81) e sadios (n= 95).

	RDW-CV (%)			
Grupos	**Média ± dp**	**Variação**	**IC 95%**	**Valor de *p***
Hospitalizados	18,6 ± 2,0	15,0-27,1	18,2-19,0	0,0006*
Sadios	17,8 ± 1,1	15,1-22,9	17,5-18,0	

dp: desvio-padrão; * teste t; IC 95%: Intervalo de Confiança de 95

Tabela 2. Influência do sexo sobre os valores de RDW-CV (%) em equinos da raça Quarto-de-Milha (QM), hospitalizados (n= 81; machos= 57 e fêmeas= 23) e sadios (n= 95; machos= 55 e fêmeas= 40).

Grupos	**Hospitalizados**		**Sadios**	
	Média ± dp (variação)	IC 95%	Média ± dp (variação)	IC 95%
Machos	$18,7^{Aa}$ ± 2,1 (15,0-27,1)	18,2-19,3	$17,7^{Ab}$ ± 0,9 (15,9-19,6)	17,4-18,0
Fêmeas	$18,4^{Aa}$ ± 1,6 (16,1-22,9)	17,7-19,1	$17,8^{Aa}$ ± 1,3 (15,1-22,9)	17,4-18,2

Letras maiúsculas diferentes em uma mesma coluna ou minúsculas diferentes em uma mesma linha indicam que houve diferença significativa entre as médias ($p<0,05$).
dp: desvio-padrão; teste t.

Tabela 3. Influência da idade sobre os valores de RDW-CV (%) em equinos da raça Quarto-de-Milha (QM) hospitalizados (n= 77) e sadios (n= 95).

Grupos	**Hospitalizdos**		**Sadios**	
	Média ± dp (variação)	IC 95%	Média ± dp (variação)	IC 95%
Idade				
< 2 anos	$20,0^{Aa}$ ± 2,5 (17,5-27,1)	18,5-21,4	$17,9^{Ab}$ ± 1,9 (15,9-22,9)	16,6-19,2
2 a 10 anos	$18,2^{Ba}$ ± 1,5 (15,0-23,0)	17,8-18,6	$17,7^{Ab}$ ± 1,0 (15,1-20,0)	17,7-17,5
> 10 anos	$18,9^{ABa}$ ± 2,3 (16,4-25,9)	16,7-21,0	$17,5^{Aa}$ ± 0,8 (16,7-19,0)	17,0-18,0

Letras maiúsculas diferentes em uma mesma coluna ou minúsculas diferentes em uma mesma linha indicam que houve diferença significativa entre as médias ($p<0,05$).
dp: desvio-padrão.

2.1 ARTIGO CIENTÍFICO 1 (INGLÊS)*

Red Blood Cell Distribution Width (RDW-CV) in Quarter Horses: A comparison between healthy and hospitalized animals

Abstract

RDW (Red blood cell distribution width) is a hematimetric index used to diagnose anisocytosis. In human medicine, it has been widely used as a predictor and biomarker of various diseases, but there are few comparable studies in equine medicine. We aimed to compare RDW values between healthy and hospitalized Quarter Horses (QH). A total of 176 QH were evaluated, 95 healthy animals reared on 27 farms, and 81 animals treated at a Veterinary School Hospital. Blood samples were evaluated using an automated hematological analyzer to obtain RDW. RDW values in hospitalized horses (18.6%) were significantly higher than in healthy ones (17.8%, p = 0.0006). Values from our healthy group can be used as a reference for QH. We suggest that further studies are needed to evaluate the use of RDW as a biomarker or predictor of various disorders or diseases in equines.

Keywords: equine; RDW; hematology; biomarker

Introduction

In routine clinical pathology, mean corpuscular volume (MCV) and reticulocyte count are the two main traditional criteria for the initial classification of anemic disorders [1]. In recent years, the use of automatic hematology counters has provided more accurate diagnoses and quicker analyses in addition to generating new erythrocyte measurements [2] such as red blood cell distribution width coefficient of variation (RDW-CV, hereafter RDW). Through quantitative analysis, this metric reflects the degree of heterogeneity among red blood cells, representing the coefficient of erythrocyte anisocytosis [3,4,5].

In recent studies on humans, RDW has been suggested as both a marker of chronic inflammatory processes and a predictor of various diseases [4,6,7,8,9]. For example, Kara et al [9] found that patients with stroke symptoms within 24 hours of injury presented significantly higher RDW than did those who sustained milder damage, demonstrating the value of RDW to predict severity and functional outcomes of stroke.

Obtaining the RDW is simple, accessible, and inexpensive [6], making it a useful tool with multiple clinical applications [10]. Despite the growing interest in this diagnostic tool in human medicine [11], studies on RDW in animals are still scarce.

In the equine clinic, RDW has been used to evaluate the relationship between size variation among red blood cells and either the intensity of exercise performed by the animals [12,13], or certain types of diets [14,15]. However, we are not aware of any studies evaluating either the relationship between RDW and the clinical state in equines, or the standardization of RDW for particular breeds such as Quarter Horses (QH).

Given the importance of RDW as a predictor of inflammatory processes and other injuries in humans, and the lack of corresponding information for horses, we aimed to compare RDW values between healthy QH and QH attended at a Veterinary Hospital School.

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Material and Methods

Study population

This study was carried out from March 2016 to June 2017. Blood samples from 176 QH (95 healthy horses and 81 hospitalized horses) were evaluated.

Regardless of sex, age, and type of husbandry, healthy QH were selected from 14 properties visited in the study region and their health was evaluated by clinical examination following a previously-described protocol [16]. Blood samples (10 ml) were then collected by jugular venous puncture in a vacuum tube with 10% ethylenediaminetetraacetic acid (EDTA) anticoagulant for complete blood counts.

Data for hospitalized QH were obtained from hematological records from 2016 to 2017 in the Veterinary School Hospital. Horses presenting infectious/inflammatory processes or under postoperative period were included in the study.

Sample analysis

Blood samples were processed in an automatic analyzer (pocH-100iV-DIFF, Sysmex Roche Corporation, Japan) to obtain hematological values, including the width of the erythrocyte size distribution (RDW-CV).

RDW values were compared between hospitalized and healthy animals, and the influence of sex and age of the animals was evaluated.

Data analyses

All statistical analyses were performed using the statistical software GraphPad Prism (version 7.04, GraphPad Software Inc., La Jolla, USA). RDW values were tested for normality. Student's t-test and ANOVA were used to evaluate the influence of sex and age on RDW, respectively. Correlations between RDW and other hematological parameters (hematocrit and MCV) were assessed using Pearson's correlation. The chi-squared test was used to evaluate the association between type of husbandry and equine activity among QH with RDW elevation. Values of $p < 0.05$ were considered significant.

Results

Our study population of 81 hospitalized QH represented 71.7% of the horses treated in the Veterinary Hospital (n = 113) in 2016 and 2017. Mean RDW values for hospitalized QH were 4.5% higher than those observed in healthy horses (18.6 and 17.8 respectively, $p = 0.0006$, Figure 1).

Among hospitalized animals, RDW was negatively correlated with both hematocrit ($r = -0.3634$, $p < 0.0009$) and MCV ($r = -0.2882$, $p < 0.0091$), but this was not true for healthy animals. We observed a positive association (odds ratio = 3.115, $p = 0.0333$, 95% CI = 1.133 - 8.563) between the presence of anisocytosis and RDW values above those considered normal in the present study (17.7%).

Seven horses in the hospitalized group were admitted for castration, and the RDW was analyzed one day after the surgical procedure. The mean RDW values for these horses were indistinguishable from those observed for the other hospitalized horses (RDW = 18.6%, range 16.0 - 19.1).

Within each of the two groups (hospitalized and healthy), mean RDW did not differ between sexes. However, mean RDW was significantly higher for hospitalized than for healthy males, a difference that was absent for females (Table 1).

Age was unrelated to the RDW of healthy animals, but among hospitalized animals less than two years old RDW was higher than in animals aged 2 to 10 years. Mean RDW values for both these age groups were higher than those for healthy animals (Table 2).

The RDW values of stabled animals (17.8 ± 0.8%) were indistinguishable from those of animals kept in the field (17.8 ± 1.2%), conforming to the mean observed in the healthy QH population. There were no differences between RDW values of animals used for different activities (sports, work, or leisure).

Discussion

The present study demonstrates that RDW values in hospitalized animals were significantly higher than in healthy animals. First, we will consider number of factors other than hospitalization that might influence RDW in the QH.

The mean RDW of 17.8% that we report for healthy QH is identical to that observed for the same breed before physical activity (also 17.8%, n = 29) [13], but was only 63% of the value from another study (28.4%) [17]. The discrepancy is probably due to the automatic analyzer: we used the same model as did Miranda et al [13], which was different to that used by Carvalho et al [17]. The analyzer model that we used is considered excellent for evaluation of hematological data of horses [18].

Counting patterns differ between different models of hematological analyzers, influencing measurement of RDW in dogs [19]. Similar observations in human studies have led to the recommendation that use of the same hematological analyzer model is a prerequisite for accurate comparisons [20]. This source of variation is a serious limitation for the establishment of universal RDW reference values [10].

RDW in healthy equines is higher than the range for other species [21], and probably differs between horse breeds [22]. The mean RDW that we observed was lower than that reported for other horse breeds such as Holsteiner (range: 20.3% to 36.1%) [23,24], Thoroughbred (26.9% to 27.3%) [12,25], and Mangalarga Marchador (18.8% to 20.4%) [14,15,22,26]. Hematological evaluations for the Mangalarga Marchador horses used the same equipment as our study, while those of Holsteiner and Thoroughbred horses used different models. Balarin et al [12] assessed the effect of both breed and laboratory protocol on MCV and RDW, and emphasized the importance of establishing reference hematimetric values for each region and breed.

Husbandry conditions may also be an important determinant of hematological reference ranges for horses [22]. We assessed this effect using within-group comparisons between horses that were stabled or kept in the field, and between activity levels (work, sport, or leisure), and found no influence of these factors on RDW.

Carvalho et al [17] reported no significant differences in MCV and RDW between males and females. It is worth noting that their study was carried out with animals during physical activity, which has a strong influence on MCV [27].

After accounting for the above factors, we aimed to compare RDW between healthy and hospitalized animals. We report higher mean values in hospitalized than in healthy QH. RDW is an excellent indicator for the classification of anemia [3,4]. We observed an association between anisocytosis and RDW values above the

mean of healthy animals, and a negative correlation between RDW and hematocrit and MCV values only in the hospitalized animals.

In humans, RDW has been described as a prognostic predictor of various diseases including several types of neoplasms [5,11,28,29], chronic myeloproliferative diseases [30], Chronic Obstructive Pulmonary Disease (COPD) [31], aneurysms [4], and cardiac pathologies [3,32,33]. For example, it was observed that for every 1% increase in RDW there was an 11% increase in the risk of mortality from venous thromboembolism in human patients [4]. In our study, mean values in hospitalized horses were 4.5% above those of healthy animals. The hospitalized animals we studied had diverse causes of hospitalization, with some frameworks of infectious/inflammatory processes, which may have influenced this increase in RDW.

We assessed the influence of sex and age on RDW values. Balarin et al [24] postulated that RDW reference values can be used independently of the sex of animals, which is corroborated by the results of other equine studies [15,22], and also by our study, which found that sex did not influence RDW within either of the study groups.

In the present study, mean RDWs did not differ between ages within the healthy animal group. In contrast, hospitalized animals less than two years old had higher RDW than did animals aged 2 to 10 years. In a study with Mangalarga Marchador horses, RDW values were significantly higher in animals aged 2 to 6 years, in relation to older animals [22]. However, these authors did not evaluate animals less than 2 years old, preventing direct comparison with our results but showing that young animals may present RDWs higher than reference values for the general population. Alis et al [20] verified the importance of age as a confounding factor in the interpretation of RDW in humans.

Balarin et al [12] observed a significant increase in RDW in horses after exercise of different intensities. Other studies have also reported this increase after exercise [14,15]. In the study by Miranda et al [13], RDW values in horses remained unchanged after physical activity. The authors attributed this result to a likely minimal release of cells by the spleen, insufficient to cause great heterogeneity among erythrocytes. In our study both the hospitalized and field animals were at rest, so the increase in RDW that we report in the hospitalized animals was not due to physical activity.

Sample size is one of the main limitations of RDW studies on horses and other animal species. Human studies are often conducted on large datasets that allow retrospective or prospective analysis of information with increased power of RDW as an indicator of morbidity or predictor of various diseases.

Comparison between our results and those of other studies allows us to infer that variation in RDW reference values may occur between breeds and be influenced by the equipment used to perform the hematological analysis.

Although the present study is preliminary, our data show that RDW values in hospitalized animals are significantly higher than in healthy animals, and we suggest that further studies are needed to evaluate the use of RDW as a biomarker or predictor for various disorders or diseases in horses.

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Tble 1. RDW-CV mean values (%) in Quarter-Horse (QM), hospitalized (n = 81) and healthy (n = 95).

	RDW-CV (%)			
Groups	Mean± sd	Range	CI 95%	*P value*
Hospitalized	18,6 ± 2,0	15,0-27,1	18,2-19,0	0,0006*
Healthy	17,8 ± 1,1	15,1-22,9	17,5-18,0	

sd: standard deviation; *t test; confidence interval of 95%

Table 2. Influence of sex on the values of RDW-CV (%) in hospitalized (n = 81, males = 57 and females = 23) and healthy (n = 95, males = 55 and females = 40) Quarter horses.

Groups	**Hospitalized**		**Healthy**	
	Mean ± sd(variation)	95%CI	Mean ± sd(variation)	95%CI
Males	18.7^{Aa} ± 2.1 (15.0-27.1)	18.2-19.3	17.7^{Ab} ± 0.9 (15.9-19.6)	17.4-18.0
Females	18.4^{Aa} ± 1.6 (16.1-22.9)	17.7-19.1	17.8^{Aa} ± 1.3 (15.1-22.9)	17.4-18.2

Different capital letters in the same column or different lowercase letters in the same row indicate that there was a significant difference between the means ($p < 0.05$).
sd: standard deviation.
CI: Confidence Interval.

Table 3. Influence of RDW-CV (%) on hospitalized (n = 77) and healthy (n = 95) Quarter horses.

Groups	**Hospitalized**		**Healthy**	
	Mean ± sd (range)	95%CI	Mean ± sd (range)	95%CI
Age				
< 2 years	20.0^{Aa} ± 2.5 (17.5-27.1)	18.5-21.4	17.9^{Ab} ± 1.9 (15.9-22.9)	16.6-19.2
2 to 10 years	18.2^{Ba} ± 1.5 (15.0-23.0)	17.8-18.6	17.7^{Ab} ± 1.0 (15.1-20.0)	17.7-17.5
> 10 years	18.9^{ABa} ± 2.3 (16.4-25.9)	16.7-21.0	17.5^{Aa} ± 0.8 (16.7-19.0)	17.0-18.0

Different capital letters in the same column or different lower case letters in the same row indicate that there was a significant difference between the means ($p < 0.05$).
sd: standard deviation.
CI: Confidence Interval.

3 ARTIGO CIENTÍFICO II*

RDW-CV como biomarcador para infecção por estrongilídeos em equinos

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Resumo

O RDW-CV (amplitude de distribuição dos glóbulos vermelhos) tem sido considerado um biomarcador / preditor fácil de se obter, de baixo custo, rápido e eficaz para diferentes doenças humanas, incluindo um grande número de neoplasias. Em razão do RDW-CV ser empregado como um biomarcador de isquemia mesentérica aguda em humanos que apresentam dor abdominal, nós hipotetizamos que o RDW-CV poderia também ser usado como um biomarcador para infecção por *Strongylus vulgaris*. Foram avaliados 160 equinos sadios (Quarto de Milha e mestiço de Quarto de Milha), com pelo menos dois anos de idade e de ambos os sexos. O ELISA apresentou resultado positivo em 80% das amostras, no entanto, a coprocultura e a PCR específica foram negativas para *S. vulgaris*, apesar do elevado número de animais (91,25%) positivos para contagem de ovos (> 50 epg). Nenhuma evidência de associação foi observada entre RDW-CV e teste ELISA. Estes resultados são discutidos e, apesar da falta de associação, mais investigações são necessárias para avaliar o papel do RDW como biomarcador/preditor de arterite verminótica em equinos apresentando cólica.

Palavras-chave: RDW; cólica; diagnóstico; cavalo; arterite verminótica.

RDW as a biomarker of infection by Strongylidae in horses

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Abstract

RDW-CV (red cell distribution width) has been considered an easy, low cost, quick, and effective biomarker/predictor for different human disorders, including a wide number of cancers. As RDW-CV has been employed as a biomarker of mesenteric acute ischemia in humans presenting abdominal pain, we hypothesized that RDW-CV could be used as a biomarker for *Strongylus vulgaris* in naturally infected horses. One hundred sixty horses (Quarter horses and cross breed Quarter Horses) at least 2-years-old were evaluated in this study, independent of the sex. Stool samples were analysed for nematode egg count, and coprocultures were processed for identification of third stage larvae. Specific PCR was performed for amplification of *S. vulgaris* DNA, and the ELISA test was run to detect anti-*S. vulgaris* antibodies. No association was observed between RDW-CV (%) and epg or for the ELISA. In our study, the ELISA tested positive in 80% of samples, however, despite the high number of animals (91.25%) positive for egg count (>50 epg), the coproculture and PCR were negative for *S. vulgaris*. These results were discussed and, it was concluded that further investigations are necessary to evaluate the role of RDW as a biomarker for verminotic arteritis.

Keywords: colic; diagnosis; equine; verminotic arteritis.

1. Introdução

A análise automatizada baseada na citometria de fluxo forneceu uma determinação mais rápida e precisa dos parâmetros hematológicos. Além disso, a automaticidade gerou novos parâmetros para a discriminação de anemias, particularmente RDW (amplitude de distribuição dos glóbulos vermelhos), um índice que determina o grau de homogeneidade/heterogeneidade das células eritrocitárias.

Nos últimos anos, o RDW foi adotado na medicina como um biomarcador/preditor de fácil obtenção, barato, rápido e forte para uma ampla gama de distúrbios humanos, como o cardíaco (Breda et al., 2015; Duchnowski et al., 2016; Ellingsen et al., 2016; al, 2018), hepático (Lou et al., 2017), doença renal crônica (Yonemoto et al., 2018), síndrome metabólica pulmonar (Breda et al., 2015), (Jo et al., 2013; Hacibekiroglu, 2015), e acidente vascular cerebral isquêmico (Riedl et al., 2014; Kara et al., 2015; Keser et al., 2016), bem como para o prognóstico de uma variedade de neoplasias, incluindo de mama (Seretis et al., 2013; Huang et al., 2016), colorretal (Ay et al, 2015), esofágica (Xu et al., 2018), gástrica (Cheng et al., 2017), mieloma (Lee et al., 2014), linfoma (Zhou et al., 2017), pulmonar (Koma et al., 2013), pancreática (Yilmaz et al., 2014), prostática (Albayrak et al., 2014), renal (Wang et al., 2014) da tireoide (Aktas et al., 2017), entre outros.

RDW também tem sido apontado como um parâmetro sensível e barato para o diagnóstico de isquemia aguda mesentérica em humanos (Kisaoglu et al., 2014), uma doença fatal em 50-70% dos casos (Tanrıkulu et al., 2017). Em equinos, a isquemia mesentérica tem sido associada à cólica verminótica, uma síndrome potencialmente fatal (Pilo et al., 2012; Fabiani et al., 2016). A síndrome é desencadeada pelas larvas de quarto estágio (L4) do nematóide *Strongylus vulgaris*, o parasito intestinal mais patogênico que infecta equinos (Andersen et al., 2013; Nielsen et al., 2014; Abo-Aziza et al., 2017; Pihl et al., 2014; Stancampiano et al., 2017).

A *S. vulgaris* L4 penetra nas arteríolas do intestino e migra abaixo do endotélio para a artéria mesentérica cranial e seus ramos, levando ao depósito de fibrina e à formação de aneurismas (Duncan, 1974). Consequentemente, o tromboembolismo resultante compromete a circulação sanguínea intestinal, provocando isquemia intestinal local ou generalizada (Nielsen et al., 2012; Pilo et al., 2012; Borji et al. 2014; Nielsen et al., 2015; Nilsen et al., 2016).

O diagnóstico de *S. vulgaris* tem sido baseado na identificação morfológica de L3 em coproculturas (Andersen et al., 2013). Recentemente, métodos imunológicos e moleculares foram desenvolvidos para detectar anticorpos anti-*S. vulgaris* (Phil et al., 2017) ou DNA (Bracken et al., 2012). No entanto, há uma escassez de informações sobre o uso de biomarcadores para inflamação causada por larvas de *S. vulgaris*, incluindo o RDW.

Ao considerar estas afirmações e a importância da cólica verminótica na medicina eqüina, nós hipotetizamos que o RDW poderia ser um biomarcador confiável para *S. vulgaris* em equinos naturalmente infectados.

2. Material e métodos

2.1 Considerações éticas

O estudo foi aprovado pelo Comitê de Ética em Estudos Animais (CEUA-Protocolo) e o consentimento foi recebido dos proprietários dos animais.

2.2. Área do estudo

O estudo foi realizado no período de agosto de 2016 a janeiro de 2017. Vinte e seis propriedades localizadas na região oeste do Estado de São Paulo, Brasil, foram visitadas. A região é considerada um importante pólo criador de Quarto de Milha no Brasil.

2.3 Animais

Cento e sessenta cavalos da raça Quarto de Milha (QM) ou cavalos de raça mestiça (RM), independentemente do sexo, foram incluídos neste estudo. Como critérios de inclusão, definimos: o último tratamento altihelmíntico dos equinos ter sido realizado pelo menos 60 dias antes do início do estudo; igual ou acima de dois anos de idade; e em estado saudável.

Os animais com menos de dois anos foram excluídos do nosso estudo. Alis et al. (2016) verificaram que a idade pode ser considerada como um importante fator de confundimento na interpretação do RDW, uma vez que os animais jovens podem apresentar valores do biomarcador maiores que os considerados como de referência para a população geral.

Os plantéis que participaram desse estudo foram escolhidos de forma independente do número, manejo e uso dos equinos.

2.4 Exame clínico e coleta de amostras

O exame clínico baseou-se na temperatura retal, parâmetros cardíacos e auscultas respiratória e intestinal, de acordo com o protocolo recomendado por Blood (2000).

As amostras de fezes foram coletadas diretamente da ampola retal dos equinos utilizando uma luva de palpação. Estas amostras foram armazenadas em sacos plásticos e mantidas sob refrigeração até o processamento.

O sangue foi coletado por punção jugular usando tubos a vácuo. Uma amostra de 5,0 mL foi coletada em um tubo contendo EDTA como anticoagulante para realizar a contagem sangüínea (incluindo RDW), e outros 5,0 mL coletados em um tubo sem anticoagulante para obtenção da amostra de soro para o teste ELISA. As amostras foram acondicionadas em caixa térmica com gelo reciclável até serem levadas ao laboratório. Todas as amostras, fezes e sangue, foram processadas no mesmo dia da coleta.

2.5 Processamento das amostras

2.5.1 Análise hematológica

Os parâmetros hematológicos, incluindo RDW-CV (%), foram obtidos utilizando um analisador hematológico semiautomático (Sysmex pocH-100iVTM, Roche®, Japão).

2.5.2 Contagem de ovos por grama de fezes

A contagem de ovos por grama de fezes (OPG) de todas as amostras foi determinada usando a técnica McMater modificada com um limite de detecção de 50 OPG (Renemeyer; Nielsen, 2013). Cada amostra foi analisada em triplicata, e o resultado final foi obtido através da média das três contagens.

2.5.3 Recuperação e identificação de larvas de *Strongylus vulgaris*

A técnica de Roberts e O'Sullivan (1950), modificada, foi utilizada para recuperar larvas de estrongilídeos. Três gramas de fezes foram misturadas e incubados por 14 dias à temperatura ambiente. Após o período de incubação, foi utilizada a microscopia óptica (objetivas 10x e 40x) para contagem e identificação das larvas de acordo com a chave de classificação (Russel, 1948; Bevilaqua et al., 1993).

2.5.4 PCR específico para *Strongylus vulgaris*

As larvas recuperadas das coproculturas foram analisadas quanto à presença de DNA de *S. vulgaris* seguindo um protocolo de PCR convencional (Bracken et al., 2012).

Resumidamente, o DNA genômico das larvas foi extraído usando um Kit comercial PureLink (Invitrogen K182001, Califórnia, EUA), seguindo o protocolo do fabricante. Os primers utilizados em nosso estudo foram desenvolvidos e validados por Nielsen et al. (2008). Um controle negativo e um positivo foram incluídos para cada teste de PCR. O controle positivo consistiu de DNA extraído de parasitos adultos identificados morfologicamente como *S. vulgaris*.

Após ativação enzimática por 2 min a 95ºC, 40 ciclos foram executados, cada um consistindo de uma etapa de desnaturação a 95 ° C por 30 s, uma etapa de anelamento a

550°C por 30 s e uma etapa de extensão a 700°C por 30 s, usando um termociclador (Termociclador MultiGene™ Gradient PCR do Labnet).

Os produtos de PCR foram corridos em gel de agarose a 1% contendo brometo de etio (5/100 mL). Uma escala de DNA de 100 pb (New England Biolabs, Ipswich, MA 01938-2723, EUA) foi utilizada como marcador.

2.5.5 Anti-*Strongylus vulgaris* detecção de anticorpos (teste de ELISA)

Foi realizado um ensaio indireto de imunoadsorção enzimática (ELISA) usando a proteína recombinante SvSXP para investigar a presença de anti-anticorpos *S. vulgaris*, de acordo com o protocolo descrito por Andersen et al. (2013). As amostras foram diluídas (1:50) e o conjugado anti-IgG equino produzido em caprino marcado com peroxidade (Bethyl Laboratories, Inc., Montgomery, TX, USA) foi usado como anticorpo secundário na diluição 1:40.000. Os resultados foram obtidos como valor normalizado, a porcentagem do controle positivo foi usada para reduzir a variabilidade inter-ensaio (Andersen et al., 2013).

O controle positivo para o teste de ELISA foi obtido a partir da amoatra de soro de um cavalo mantido em um plantel de eqinos naturalmente infectados com *S. vulgaris* (Universidade de Kentucky, Lexington, EUA). Esses equinos eram mantidos sem tratamento anti-helmíntico por décadas (Nielsen et al., 2014) e infecção confirmada através de exame necroscópico (Fabiani et al., 2016).

Todas as amostras de soro foram testadas em duplicata. O teste ELISA foi considerado positivo com base no limite do título de 13,47 % do controle positivo (Andersen et al., 2013).

Análise dos Resultados

Os valores da contagem média do OPG e ELISA foram submetidos ao teste de Spearman para avaliar a possível relação com o RDW-CV (%), tanto para os 160 cavalos quanto para os grupos QM e RM separadamente.

RDW-CV (%) em QM e RM foram comparados pelo teste t. O mesmo teste foi empregado para comparar a contagem de OPG observada nos dois grupos.

A associação entre o ELISA e o RDW-CV (%) foi avaliada com o uso do teste Kappa, considerando-se os animais positivos, com base em dois pontos de corte para o RDW-CV (%). Um deles, testado com a média obtida (17,8) e o outro com a média acrescida do desvio-padrão (17,8 + 1,1=18,9).

Um nível de significância bicaudal de 0,05 foi utilizado para avaliar a significância estatística.

3. Resultados

Neste estudo foram avaliados 160 cavalos, sendo 92 da raça QM e 68 de RM. O RDW-CV foi idêntico nos dois grupos (média = 17,75% ± 1,0), como mostra a Tabela 1.

A contagem positiva de OPG (≥ 50 OPG) foi verificada em 91,25% dos equinos. Deste total, 83,8% (57/68) eram do grupo RM e 33,7% (31/92) do grupo QM. A contagem de ovos por grama de fezes nos animais QM (média = 925,93) foi significativamente maior ($p = 0,0057$) do que o observado nos animais de RM (média = 554,5).

As larvas de *S. vulgaris* não foram recuperadas nas coproculturas, nem o DNA amplificado por PCR. Assim, utilizamos os resultados do ELISA para considerar a infecção / contato de equinos com *S. vulgaris*.

Não foi observada associação entre as contagens de RDW-CV e OPG ($p = 0,9843$; $r^2 = 0,0016$).

O teste ELISA mostrou que 80,0% (128/160) dos equinos foram positivos para anticorpos contra *S. vulgaris*. Considerando-se os animais positivos (n=128), verificou-se que 58,6% (75/128) eram QM e 41,4% (53/128) RM.

Os valores de RDW de 17,8% (média) e 18,9% (média ± 1,1) foram usados para testar a associação entre RDW e o teste ELISA positivo, considerando 160 animais ou avaliando os grupos QM e RM separadamente. De acordo com nossos resultados, o RDW-CV não foi associado ao teste ELISA (Tabela 2).

4. Discussão

O uso do RDW como biomarcador em Medicina Veterinária está restrito a um número pequeno de estudos em cães (Swann et al., 2014; Mazzotta et al., 2016; Braz et al., 2017). Nos equinos há uma escassez de informações sobre o papel do RDW como biomarcador de doenças.

Neste estudo, nós hipotetizamos a possibilidade do RDW ser empregado como um biomarcador para o diagnóstico de *S. vulgaris* em equinos QM e RM naturalmente infectados. Nossos dados demonstraram falta de evidências para esse fim. O RDW-CV não foi associado ao teste ELISA ou à contagem de OPG.

Apesar da coprocultura e PCR para *S. vulgaris* serem negativos, o teste ELISA revelou a presença de anticorpos *S. vulgaris* em 80,0% dos equinos. A cultura de larvas tem sido considerada como ferramenta de diagnóstico para detectar infecções por *S. vulgaris*, mas estas não refletem as larvas migratórias patogênicas durante o período pré-patente de seis meses (Andersen et al., 2013; Nielsen et al., 2014).

As culturas analisadas em nosso estudo foram predominantemente de larvas de ciatostomíneos, como observado em outros estudos (Matthews, 2014; Tzelos et al., 2017; Scare et al., 2018). Portanto, os testes negativos de PCR encontrados em nosso estudo não foram um resultado surpreendente. O uso indiscriminado de antihelmínticos em equinos levou ao surgimento de cepas resistentes à ciatostomíenos (Samson-Himmelstjerna, 2012), inclusive no Brasil (Molento, 2008; Canenver et al., 2013). Como consequência, enquanto os

ciatostomíenos sobrevivem ao antihelmíntico, o parasito adulto de *S. vulgaris* é suscetível à desverminação (Lyons et al., 2000; Nielsen et al., 2014).

Pesquisas e necropsias realizadas em cavalos manejados em diferentes partes do mundo continuam a documentar que o *S. vulgaris* ainda é encontrado regularmente (Veiga et al., 2011; Pilo et al., 2012; Nielsen et al., 2014; Fabiani et al., 2016; Pihl et al., 2017).

O longo ciclo de vida do *S. vulgaris* de seis meses e meio e a longa persistência dos níveis de anticorpos por até cinco meses após a desverminação (Nielsen et al., 2014) podem indicar que um ELISA positivo é uma exposição atual ou recente à migração de larvas de *S. vulgaris*, enquanto a PCR e a coprocultura sugerem uma falta geral de estágios adultos patentes (Nielsen et al., 2016). A principal limitação do nosso estudo foi a avaliação de equinos naturalmente infectados e saudáveis. Pode ser possível considerar que, apesar dos resultados negativos da coprocultura e da PCR, os equinos podem ser infectados continuamente por larvas de *S. vulgaris* presentes em pastagens, como observado em outros estudos (Reinemayer e Nielsen, 2013).

O diagnóstico laboratorial de infecção patente por *S. vulgaris* é um desafio para os veterinários (Andersen et al., 2013), pois as infecções de longa duração podem causar cólicas intermitentes em casos não fatais, que são muito difíceis de diagnosticar (Owen e Slocombe, 1985, Pilo et al., 2012). Algumas técnicas têm sido consideradas para o diagnóstico antemortem de arterite verminótica, incluindo ultrassonografia transretal e radiografia (Wallace et al., 1989; Veiga et al., 2011), mas os métodos têm algumas limitações, incluindo alto custo e experiência clínica (Nielsen, 2013).

O conhecimento da epidemiologia da cólica, juntamente com a interpretação dos resultados do exame clínico e testes diagnósticos para auxiliar na tomada de decisões, são essenciais. O diagnóstico precoce e o tratamento cirúrgico da cólica verminótica permanecem cruciais para aumentar a sobrevida do equino (Archer, 2017). Porém, na maioria das vezes, a

intervenção cirúrgica ocorre quando o animal está em estágio avançado do processo de tromboembolismo, o que torna o prognóstico reservado (Borj et al., 2014). Por esta razão, vale a pena investigar um preditor de baixo custo, fácil e rápida obtenção como o RDW, para processos inflamatórios agudos, incluindo aqueles desencadeados por *S. vulgaris*.

Embora nossos resultados não sejam promissores para a associação do RDW com *S. vulgaris* em equinos saudáveis, o RDW tem sido considerado um biomarcador confiável para o diagnóstico de isquemia mesentérica aguda em humanos (Kisaoglu et al., 2014; Bilgiç et al., 2015). O processo inflamatório na isquemia intestinal, devido à disfunção causada por várias alterações bioquímicas, metabólicas e ultra-estruturais nas células epiteliais, resulta em morte celular e pode causar alterações hematológicas e bioquímicas no sangue (Grosche et al., 2013). Esses eventos são semelhantes aos observados em humanos (Eltzsching; Eckler, 2011). A liberação de citocinas inflamatórias altera a eritropoiese, resultando em altos níveis de RDW (Riedl et al., 2014).

Mais estudos com equinos são necessários para plotar uma curva ROC para avaliar a sensibilidade e especificidade do RDW, conforme observado em estudos com humanos (Kisaoglu et al., 2014; Bilgiç et al., 2015; Tanrıkulu et al., 2017). Uma das possibilidades é a avaliação do RDW em equinos de matadouros apresentando arterite intestinal causada por larvas de *S. vulgaris*. Sugere-se um estudo que vise a avaliação do RDW em equinos com sinais clínicos de síndrome cólica durante o tratamento medicamentoso e/ou cirúrgico.

5. Conclusão

Nas condições deste estudo o RDW não foi um biomarcador confiável para *S. vulgaris* em equinos naturalmente infectados.

Conflito ou declaração de interesse

Os autores declaram não ter interesses conflitantes.

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Tabela 1. RDW-CV (%) valores em equinos QM adultos saudáveís (QM; n = 92) e mistos da raça QM (RM; n = 68).

QM		**RM**		
Média ± dp(variação)	IC 95%	Média ± dp(variação)	IC 95%	*p*-value
17,75 ± 1,14 (15,1 - 22,9)	17,52 - 17,99	17,75 ± 1,01 (15,5 - 20,3)	17,51 - 18,0	0,990*

dp: desvio padrão; * teste t

Tabela 2. Associação entre RDW-CV (teste 1 = média obtida = 17,8; e teste 2 = média ± dp = 17,8 ± 1,1 = 18,9) e anticorpos anti-Strongylus vulgaris (teste de ELISA) em adultos de QM e mestiços QM (n = 160) cavalos.

		Teste de ELISA			
		Positivo (%)	**Negativo (%)**	**Kappa**	**Valor de *P***
	RDW-CV(%)				
	Teste 1				
Positivo	≥ 17,8	55 (34,4)	13 (8,1)	-	
Negativo	<17,8	73 (45,6)	19 (11,9)	1,101 (0,5009-2,421)	0,9681*
	Teste 2				
Positivo	≥ 18,9	22 (13,7)	3 (1,9)	-	
Negativo	< 18,9	106 (66,3)	29 (18,1)	2,006 (0,5608-7,177)	0,4147**

3.1 ARTIGO CIENTÍFICO 2 (INGLÊS)*

RDW as a biomarker of infection by Strongylidae in horses

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Abstract

RDW-CV (red cell distribution width) has been considered an easy, low cost, quick, and effective biomarker/predictor for different human disorders, including a wide number of cancers. As RDW-CV has been employed as a biomarker of mesenteric acute ischemia in humans presenting abdominal pain, we hypothesized that RDW-CV could be used as a biomarker for *Strongylus vulgaris* infection. We evaluated 160 healthy horses (Quarter Horses and cross breed Quarter Horses) at least 2-years-old, independent of the sex. The ELISA tested positive in 80% of samples, however, the coproculture and specific PCR were negative for *S. vulgaris*, despite the high number of animals (91.25%) positive for egg count (>50 epg). No evidence of association was observed between RDW-CV and ELISA test. These results are discussed and, despite the lack of association, further investigations are necessary to evaluate the role of RDW as a biomarker/predictor for verminotic arteritis in horses presenting colic.

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Keywords: RDW**;** colic; diagnosis; equine; verminotic arteritis.

1. Introduction

Automated analysis based on flow cytometry has provided faster and more precise determination of hematologic parameters. In addition, automaticity has generated new parameters for discrimination of anemias, particularly RDW (red cell distribution width), an index which determines the degree of homogeneity/heterogeneity of erythrocyte cells.

In recent years, RDW has been adopted in medicine as a simple, inexpensive, fast and strong biomarker/predictor for a wide range of human disorders, such as cardiac (Breda et al., 2015; Duchnowski et al., 2016; Ellingsen et al, 2018), hepatic (Lou et al., 2017), chronic kidney disease (Yonemoto et al., 2018), pulmonary (Breda et al., 2015), metabolic syndrome (Jo et al., 2013; Hacibekiroglu, 2015), and ischemic stroke (Riedl et al., 2014; Kara et al., 2015; Keser et al., 2016), as well as for prognosis of a variety of types of cancer, including breast (Seretis et al., 2013; Huang et al., 2016), colorectal (Ay et al, 2015), esophageal (Xu et al., 2018), gastric (Cheng et al., 2017), myeloma (Lee et al., 2014), lymphoma (Zhou et al., 2017), pulmonary (Koma et al., 2013), pancreatic (Yilmaz et al., 2014), prostatic (Albayrak et al., 2014), renal (Wang et al., 2014), and thyroid (Aktas et al., 2017), among others.

RDW has also been pointed out as a sensitive and inexpensive parameter for the diagnosis of mesenteric acute ischemia in humans (Kisaoglu et al., 2014), a disease fatal in 50-70% of cases (Tanrıkulu et al., 2017). In horses, mesenteric ischemia has been associated with verminotic colic, a potentially fatal syndrome (Pilo et al., 2012; Fabiani et al., 2016). The syndrome is triggered by the fourth stage larvae (L_4) of the nematode *Strongylus vulgaris*, the most pathogenic parasite infecting horses (Andersen et al., 2013; Nielsen et al., 2014; Abo-Aziza et al., 2017; Pihl et al., 2017; Stancampiano et al., 2017). *S. vulgaris* L_4 penetrates into the intestine arterioles and migrates subendothelially to the cranial mesenteric artery, leading to the deposit of fibrin and the formation of aneurysms (Duncan, 1974). Consequently, the resulting thromboembolism compromises the intestinal blood circulation, provoking local or generalized intestinal ischemia (Nielsen et al., 2012; Pilo et al., 2012; Borji et al. 2014; Nielsen et al., 2015; Nilsen et al., 2016).

Diagnosis of *S. vulgaris* has been widely based on the morphological identification of L_3 in coprocultures (Andersen et al., 2013). Recently, immunological and molecular methods have been developed to detect anti-*S. vulgaris* antibodies or DNA, amplifying/quantifying, respectively (Phil et al., 2017). However, there is a paucity of information regarding the use of biomarkers for inflammation caused by *S. vulgaris* larvae, including RDW.

In considering these statements and the importance of verminotic colic in equine medicine, we hypothesized that RDW could be a reliable biomarker for *S. vulgaris* in naturally infected horses.

2. Material and methods

2.1 Ethical considerations

The study was approved by the Ethics Committee for Animal Studies (CEUA-Protocol) and consent was received from the owners of the animals.

2.2. Area of study

The study was carried out from August 2016 to January 2017. Twenty-six farms located in the western region of the State of São Paulo, Brazil, were visited. The region is considered an important center for Quarter Horses in Brazil.

2.3 Animals

One hundred sixty Quarter Horses (QH) or cross-breed (CB) horses, independently on sex, were included in this study. As inclusion criteria, we defined: deworming of horses at least 60 days prior to the start of the study; at least 2 years of age; and a healthy status.

Animals with less than two years old were excluded from our study. Alis et al. (2016) observed that age can be considered as an important confounding factor in the interpretation of RDW, since young animals may present biomarker values higher than those considered as reference for the general population.

The herds that participated in the study were independently chosen based on the number, management and use of the horses.

2.4 Clinical examination and sample collection

The clinical examination was based on rectal temperature, cardiac parameters and respiratory and intestinal auscultation, according to the protocol recommended elsewhere (Blood, 2000).

Stool samples were collected directly from the rectal ampoule of the horses using a new palpation glove.

Blood was collected by jugular puncture using vacuum tubes. A sample of 5.0 mL was collected in a tube containing EDTA as an anticoagulant to perform the blood count

(including RDW), and another 5.0 mL drawn in a tube free of anticoagulant to obtain the serum sample for the ELISA test.

The samples collected were placed into plastic bags and stored under refrigeration in a cooler until processing. All the samples were processed on the same day as the collection.

2.5 Processing samples

2.5.1 Hematological analysis

Hematological parameters, including RDW-CV (%), were obtained using a semi-automatic haematological analyzer (Sysmex pocH-100iVTM, Roche®, Japan).

2.5.2 Fecal egg counts

The fecal egg counts (EPG) from all stool samples were determined using a modified McMater technique with a 50 EPG detection limit (Renemeyer; Nielsen, 2013). Each sample was analyzed in triplicate, and the final result was obtained through the mean of the three counts.

2.5.3 *Strongylus vulgaris* recovery and identification

The technique of Roberts and O'Sullivan (1950), modified, was performed to recover *S. vulgaris* larvae. Three grams of feces were mixed and incubated for 14 days at room temperature (average 37 Cº). After the incubation period, optical microscopy (objective 10x and 40x) was used to count and identify the larvae according to classification key (Russel, 1948; Bevilaqua et al., 1993).

2.5.4 *Strongylus vulgaris* specific PCR

Larvae recovered from the corpocultures were analyzed for the presence of *S. vulgaris* DNA following a conventional PCR protocol, previously described (Bracken et al., 2012).

Briefly, genomic DNA from larvae was extracted using a commercial Kit PureLink genomic DNA (Invitrogen K182001, California, USA), following the manufacturer's protocol. The primers used in our study developed and validated by Nielsen et al. (2008). A negative and a positive control were included for each PCR run. The positive control consisted of DNA extracted from adults morphologically identified as adult *S. vulgaris*.

After enzyme activation for 2 min at 95^0C, 40 cycles were run, each consisting of a denaturation step at 95◦C for 30 s, an annealing step at 55^0C for 30 s, and an extension step at 70^0C for 30 s, using a thermocycler (Labnet MultiGene™ Gradient PCR Thermal Cycler).

PCR products were run on 1% agarose gel containing ethidium bromide (5 µL/100 mL). A 100 bp DNA ladder (New England Biolabs, Ipswich, MA 01938-2723, USA) was used as a marker.

2.5.5 Anti-*Strongylus vulgaris* antibody detection (ELISA test)

An indirect enzyme linked-immunosorbent assay (ELISA) using recombinant SvSXP protein as antigen was performed to assess the presence of anti-*S. vulgaris* antibodies, according to the protocol previously described (Andersen et al., 2013). Samples were diluted 1:50 and horseradish peroxidase-conjugated goat anti-horse IgG (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used as a secondary antibody at dilution of 1:40,000. Results were reported as the normalized value, percentage of positive conytol to reduce inter-assay variability (Andersen et al., 2013).

The positive control sample was obtained from a horse kept with a herd of horses naturally infected with *S. vulgaris* (University of Kentucky, Lexington, US), which had not been dewormed for decades (Nielsen et al., 2014) and infection was confirmed through necroscopic examination (Fabiani et al., 2016).

All serum samples were tested in duplicate. The ELISA test was considered positive based on the titer threshold of 13.47 percent of the positive control (Andersen et al., 2013).

Statistical analysis

Epg mean count and ELISA values were log transformed for normalization of the data. Next, the values were submitted to the Spearman test for evaluation of the association with RDW-CV (%), both for the 160 horses and for the QH and CB groups separately.

RDW-CV (%) in QH and CB were compared by the t tes67t. The same test was employed to compare the epg count observed in the two groups.

For further analysis, the chi-square test was adopted to evaluate the association between ELISA and RDW-CV (%) using the mean± standard deviation values.

A two-tailed significance level of 0.05 was used to evaluate statistical significance.

3. Results

In this study we evaluated 92 QH and 68 CB horses. The RDW-CV was identical in both groups (mean= 17.75% ±1.0) as shown in Table 1.

The positive epg count (>50 epg) was verified in 91.25% of the horses: 83.8% (57/68) of the CB and 33.7% (31/92) of the QH. The Strongylidae epg in the QH (mean= 925.93) was significantly higher (p= 0.0057) than observed in the CB (mean= 554.5).

S. vulgaris larvae were not recovered in the coprocultures, nor DNA amplified by PCR. Thus, we used the ELISA results to consider the infection/contact of horses with *S. vulgaris*.

No association was observed between RDW-CV and epg counts (p= 0.9192; rs= 0.0081) or the ELISA test (p= 0.9192; rs= 0.0081), considering both all the horses and the two groups separately.

The ELISA test showed that 80.0% (128/160) of the horses were positive for anti-*S. vulgaris* antibodies, 58.6% (75/128) of the QH and 41.4% (53/128) of the CB.

The RDW values of 17.8% (mean) and 18.9% (mean±1.0) were used to test the association between RDW and the positive ELISA test, considering 160 animals or evaluating the QM and CB groups separately. According to our results, RDW-CV was not associated with the ELISA test (Table 2).

4. Discussion

The use of RDW as a biomarker in Veterinary Medicine is restricted to a scarce number of studies in dogs (Swann et al., 2014; Mazzotta et al., 2016; Braz et al., 2017). In equines, there is a paucity of information regarding the role of RDW as a biomarker for diseases.

In this study, we hypothesized the possibility of RDW being employed as a biomarker for the diagnosis of *S. vulgaris* in naturally infected QH and CB horses. Our data demonstrated lack of of evidence for this purpose. RDW-CV was not associated with the ELISA test or the epg count.

It was verified that, the majority of the evaluated horses being highly infected (up to 500 epg) with intestinal parasites according to other authors (Reinemeyer and Nielsen, 2013; Nielsen et al., 2018). Despite coproculture and PCR for *S. vulgaris* were negative, ELISA test revealed the presence of anti-*S. vulgaris* antibodies in 80.0% of the horses.

CR and larval culture have been considered useful as diagnostic tools to detect patent infections with *S. vulgaris* but these do not reflect the pathogenic migrating larvae during their six-month long prepatent period (Andersen et al., 2013; Nielsen et al., 2014). The cultures analyzed in our study were predominantly populated by cyathostomin larvae, as observed in

other studies (Tzelos et al., 2017; Scare et al., 2018). Therefore, the negative PCR tests performed in our study were not a surprising result. The indiscriminate use of anthelmintic in horses has led to the emergence of resistant strains of cyathostomin (Samson-Himmelstjerna, 2012; Matthews, 2014), including in Brazil (Molento, 2008; Canenver et al., 2013). As a consequence, whereas cyathostomin survive to anthelmintic, adult *S. vulgaris* are susceptible to the deworming (Lyons et al., 2000; Nielsen et al., 2014). Surveys and necropsies performed in managed horses in different parts of the world, continue to document that *S. vulgaris* is still encountered on a regular basis (Veiga et al., 2011; Pilo et al., 2012; Nielsen et al., 2014; Fabiani et al., 2016; Pihl et al., 2017).

The long life cycle of *S. vulgaris* of six months and a half and the long persistence of antibody levels for up to five months following deworming (Nielsen et al., 2014), may indicate that a positive ELISA is a current or recent exposure to migrating *S. vulgaris* larvae, while the PCR and coproculture suggest a general lack of patent adult stages (Nielsen et al., 2016). The major limitation of our study was the evaluation of naturally healthy infected horses. It may be possible to consider that, despite the negative results of the coproculture and PCR, the horses may be continuously infected by *S. vulgaris* larvae present on pasture as observed elsewhere (Reinemayer and Nielsen, 2013).

Laboratorial diagnosis of patent infection by *S. vulgaris* is a challenge for the veterinarian (Andersen et al., 2013). Long standing infections may cause intermittent colic in non-fatal field cases which are very difficult to diagnose (Owen and Slocombe, 1985, Pilo et al., 2012). Some techniques have been considered for antemortem diagnosis of verminous arteritis, including transrectal ultrasound and X-ray (Wallace et al., 1989; Veiga et al., 2011), but the methods have some limitations, including costs and clinicial experience (Nielsen, 2013).

Knowledge on the epidemiology of colic together with interpretation of the results of clinical examination and diagnostic tests to assist decision-making are essential. Early diagnosis and treatment of surgical forms of colic remains crucial for maximizing short- and long-term survival (Archer, 2017). Surgical intervention for colic occurs when the animal is in an advanced stage of the thromboembolism process. At this point, the prognosis may be considered poor (Borj et al., 2014). For this reason a cheap, easy, and quick predictor, such as RDW, for acute inflammatory processes, including those triggered by *S. vulgaris*, is worth investigating.

Although our results are not promising for associating RDW with *S. vulgaris* in healthy horses, RDW has been considered as a reliable biomarker for the diagnosis of acute

mesenteric ischemia in humans (Kisaoglu et al., 2014; Bilgiç et al., 2015). The inflammatory process in intestinal ischemia, due to the dysfunction caused by various biochemical, metabolic, and ultrastructural alterations in epithelial cells results in cell death and may cause hematologic and biochemical alterations in the blood (Grosche et al., 2013). These events are similar to those observed in humans (Eltzsching; Eckler, 2011). The release of inflammatory cytokines disrupts erythropoiesis, resulting in high RDW levels (Riedl et al., 2014).

Further studies with horses are necessary to plot an ROC curve to evaluate the sensitivity and specificity of RDW, as observed in studies with humans (Kisaoglu et al., 2014; Bilgiç et al., 2015; Tanrıkulu et al., 2017). One of the possibilities is the evaluation of RDW in slaughterhouse horses presenting intestinal arteritis caused by *S. vulgaris* larvae.

5. Conclusion

RDW was not a reliable biomarker for *S. vulgaris* in naturally infected horses. It is suggested a study carried out to evaluate RDW during transurgical and postoperative interventions in idiophatic cases or verminotic colic cases.

Conflict of Interest Statement

The authors declare that they have no conflicting interests.

Acknowledgements

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Table 1. RDW-CV (%) values in QH adult healthy (QH; n = 92) and mixed QH (MB; n = 68) horses.

QH		**MB**		
Mean ± sd(variation)	CI 95%	Mean ± sd(variation)	CI 95%	*p*-value
17.75 ± 1.14 (15.1 – 22.9)	17,52 - 17,99	17.75 ± 1.01 (15.5 – 20.3)	17.51 – 18.0	0.990*

sd: standard deviation; * t test

Table 2. Association between RDW-CV (test 1 = mean obtained = 17.8 and test 2 = mean ± SD = 17.8 ± 1.1 = 18.9) and anti-*Strongylus vulgaris* antibodies (ELISA test) in QM adults and crossbred QM (n = 160) horses.

		ELISA test			
		Positive (%)	**Negative (%)**	**Kappa**	***P* value**
	RDW-CV(%)				
	Test 1				
Positive	≥ 17.8	55 (34.4)	13 (8.1)	-	
Negative	<17.8	73 (45.6)	19 (11.9)	1.101 (0.5009-2,421)	0.9681*
	Test 2				
Positive	≥ 18.9	22 (13.7)	3 (1.9)	-	
Negative	< 18.9	106 (66.3)	29 (18.1)	2.006 (0.5608-7.177)	0.4147**

ANEXO 1 - NORMAS DA REVISTA: VETERINARY RESEARCH COMMUNICATIONS

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ANEXO 2 – NORMAS DA VETERINARY PARASITOLOGY

An international scientific journal and the Official Organ of the American Association of Veterinary Parasitologists (AAVP)), the European Veterinary Parasitology College (EVPC) and the World Association for the Advancement of Veterinary Pitology (WAAVP)

ISSN: 0304-4017

DESCRIPTION

This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology, Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology, and control of parasites in all domesticated animals, fall within the scope of the journal, Papers of geographically limited (local) interest which are not of interest to an international audience will not be accepted, Authors who submit papers based on local data will need to indicate why their paper is relevant to a broader readership.

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