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Determining factors and interspecific modeling for serum amyloid a concentrations in working horses, donkeys, and mules

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ABSTRACT

Serum amyloid A (SAA) is an acute phase protein and α -globulin that is produced in the liver in response to inflammatory cytokines. Considered a major acute phase protein in most domestic species, its concentrations rapidly decrease after resolution of inflammation, making SAA measurement a useful tool for monitoring the course of inflammation in an individual animal. The objective of this study was to compare SAA values in working equids including mules, donkeys and horses as well as values for different diseases at various times of the year. Samples were collected from 77 equids; 13 mules, 17 donkeys and 47 horses from sick or injured animals who presented to the clinic. Twenty-eight disease clinical diagnoses were compared with most cases being acute colitis. A highly significant differences between species was found when comparing SAA concentrations of mules to horses ($P < .001$) using Dunn comparison pairwise test. A difference ($P < .05$) was found between donkeys and horses, but not donkeys to mules ($P > .05$). The study suggests that SAA can be a helpful tool for disease diagnosis in mules and donkeys and suggests that the inflammatory response of mules maybe less than that of horses and donkeys but future studies evaluating SAA in healthy mules compared to diseased should be conducted.

1 Introduction

There are approximately 112 million working horses, mules, and donkeys scattered throughout the world (Upjohn et al., 2014); 46 million are located in the Americas, 7 million in Europe, 33 million in Asia, and approximately 25 million reside in Africa (Stringer, 2014). These working equids are the foundation of many developing countries, that contribute both directly and indirectly, by providing strength, utility and endurance to accomplish tasks and individual needs of the owner and or household. These tasks include, but are not limited to, assisting in labor/production of agricultural products and acting as a mode of transportation of goods or individuals. Often, household and agricultural responsibilities fall on women; working equids are fundamentally significant in lessening the work burden placed on them by providing draught, load-bearing power, and other outputs including put not limited to transportation and harvest assistance (Valette and Upjohn, 2014). Such arduous activities often place a burden on the overall health, welfare, and immunity to disease of these working

equids thus increasing the tendency for sickness and decreasing its capacity to work efficiently for the household. If the health of a working horse, mule, or donkey is compromised, the household will suffer from a loss of production efficiency and ensuing income (Brizgys and Brady, 2017). In addition to production and income loss, heavy physical burdens would be placed on women and children to continue tasks, such as water retrieval, that were the responsibilities of that animal.

Intervention strategies to improve the overall health and welfare of these working animals are present in numerous developing regions of the world in the form of non-governmental organizations (NGO's), government agencies, veterinary authority, charity organizations, and working equid owners. Health concerns and infectious diseases are the major constraining factor that limits the animal's ability to work productively. Unfortunately, there is a paucity of evidence present to quantify the occurrence, pervasiveness, and distribution of many infectious diseases in rural low-income countries (Stringer, 2014). Infectious diseases such as infectious diarrhea (Laatamna et al., 2015), Equine herpesvirus (Negussie et al., 2017), Equine influenza virus (Qi

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et al., 2010), bacterial pneumonia (Getachew et al., 2010), cellulitis (Singh et al., 2010) and tetanus (Getachew et al., 2016) are a few examples of infectious and or contagious diseases found in working horses, mules, and donkeys. However, due to the stoic nature and limited information on blood chemistry in mules and donkeys, often infectious diseases go undetected or undiagnosed until advanced stages become apparent and the animal cannot perform its daily tasks.

The utilization of serum amyloid A (SAA) stable side as well as blood assay analysis may offer significant opportunities for veterinarians working in developing communities, where research and technology is limited. Having a more robust medical assessment will increase the efficiency and accuracy of veterinary treatment to working equids, thus decreasing mortality rates or the length of time in which they cannot work.

In resource limited hospitals, as some in Morocco, where diagnostic tools may be limited, analysis of SAA offers a rapid, relatively cost effective and sensitive method to, determine clinical severity and to monitor response to treatment (Viner et al., 2017). Tracking and interpreting the acute phase response of hemostatic disturbances through the use of the SAA assay together with other routine blood work (such as blood chemistry, cbc, among others) will provide a clearer assessment of the animal in question. Serum amyloid A (SAA), an acute phase protein of the family apolipoproteins and produced by the liver in response to inflammation, is the body's initial inflammatory response to infection and or injury (Nolen-Walston, 2015; Satué et al., 2013). Normal plasma concentrations of SAA in horses are practically undetectable; if SAA concentrations rise, rapidly increasing in concentration from 10 to 1000 times during the acute phase response, these concentrations can indicate some form of inflammation or tissue damage. While studies have identified a range of SAA concentrations in horses, there is a lack of data presently regarding clinically/apparently sound animals' response concentrations of SAA in donkeys and mules (McLean et al., 2016). SAA is a non-specific acute phase protein where elevated concentrations may indicate disease or inflammation, trauma and stress; the level cannot indicate the cause of injury or specific disease, but it can act as an indicator of a potential threat to the body increasing the rate of response and treatment from veterinarians (Satué et al., 2013). SAA has a 24-h half-life, allowing for monitoring of effective treatment and increasing the predictability of successful treatment when continuing to monitor SAA concentrations. On this basis, SAA could be used as a screening test in mobile clinics for apparently healthy and clinically manifested animals, especially when owner reports are confusing because of the species in question being stoic and often relatively undemonstrative of pain or disease.

SAA is particularly useful to differentiate acute infection and inflammation from other more benign clinical diagnoses, as suggested when comparing uterine serous papillary cancer with other diseases such as uterine fibroids, ovarian cysts and endometrial polyp (Cocco et al., 2009). This is particularly important where owner histories are often confused and where the species in question are stoic and often relatively undemonstrative of pain or disease (Ashley et al., 2005). The socioeconomic impact of infectious or contagious diseases to impoverished communities, such as Morocco and other sub-Saharan countries, is significant. Working equids that cannot contribute to the household income, increase food insecurity, workload, and poverty for those relying on a horses, mules, or donkeys (Carpenter et al., 2017). Having a rapid and responsive indicator, such as SAA, creates the potential to detect early onset of disease in animals that notably tend to conceal signs of distress until the condition is more advanced. The use of a stable-side kit, designed to test for an acute inflammation marker (SAA), may allow for earlier detection of unhealthy mules and donkeys and establishment of the correct diagnosis, thus improving their prognosis, medical treatments, and utility.

The following study sought to develop a baseline of understanding SAA concentrations in mules, donkeys, and working horses that were presented to the American Fondouk Veterinary Hospital in Fes,

Morocco, with a variety of different disease diagnoses over a two-year period. The main aim of the study was to improve the understanding of SAA in a clinical setting and identify the effects that potential factors may have on the concentrations of SAA. First, we discriminated whether values found in diseased mules and donkeys are the same or different from what is documented in horses as a way to improve disease diagnostics. Second, we assessed the effects of factors such as the month, season, year and the disease that may affect the animal at the moment of testing. Third, we built predictive equations for each species assessing whether mules will show a lower SAA response to inflammation than horses and donkeys or not.

2 Materials and methods

Whole blood samples were collected by venipuncture of the jugular vein from 77 working equids ($n = 13$ mules, $n = 17$ donkeys and $n = 47$ horses) over a two-year period at the American Fondouk Veterinary Clinic in Fes, Morocco. As defined by Derrick et al. (2017b), our study consists of partially overlapping samples as some animals were tested more than once ($n = 23$ horses, $n = 5$ donkeys, $n = 6$ mules), some three ($n = 11$ horses, $n = 3$ donkeys) and some four ($n = 7$ horses, $n = 1$ donkeys) times, providing the fact that an animal not being tested more than once, that is producing a missing observation, occurred completely at random (MCAR), because the animal died, recovered from suffering the condition from which it was affected or left the facility (Derrick et al., 2017a). Hence, in our sample 44 observations were paired and 56 independent between the first time that the animals were tested and the second; 41 were paired between second and third testing; and 57 were paired compared to 43 independent between third and fourth testing. Fig. 1 describes the partially overlapping sample formation process. All animals received a physical and vital sign (heart rate, respiration and temperature) examination and the information was recorded by a veterinarian in the medical file for each working equid during the stay of the equids at the hospital.

Whole blood was analyzed for serum amyloid A (SAA) by using a commercially available stable-side kit (StableLab, Sligo, Ireland). SAA concentrations, obtained from the kit, were recorded in association with each equid's history, health status, and clinical diagnose. Clinical diagnose was mandatory as only those animals for which an official diagnose by a veterinarian had been issued, were included in the study. Clinical diagnose had been officially issued using routine tests for each case, and confirming animal status. Further than physical examination and vital signs. Surviving equids were classified as living (L) while those that did not survive, or were euthanized, were classified as deceased (D). Data collected for this study was a retrospective study from animals admitted into the American Fondouk Hospital and owner consent was obtained.

This study measured SAA maximal concentration expressed in mg / L at a continuous numeric level, while the factors considered in the model were categorical (sample time point, species, season, month, year, and clinical diagnose diagnosed). The study shows a summary of the levels for all the factors included in the model in Table 1. Adding factors such as the year, season or month can indirectly account for the effects of specific conditions taking place (climatological, handling, hygienical, etc.) for a certain period of time. These potential conglomerates of factors can account for the way in which animals physiologically respond to the diseases or conditions that they may be going through. Year, season or month, can act as alternatives when including multiple factors is not possible due to sample size limitations that difficult building highly complex models. No transformation was carried out over SAA maximal concentration data as the lack of normality is not likely to be due to the measurement method, but the measure itself being non normally distributed in the sample given its nature or the number of observations. All the data available ($n = 131$ observations from 77 animals) was tested to check whether data

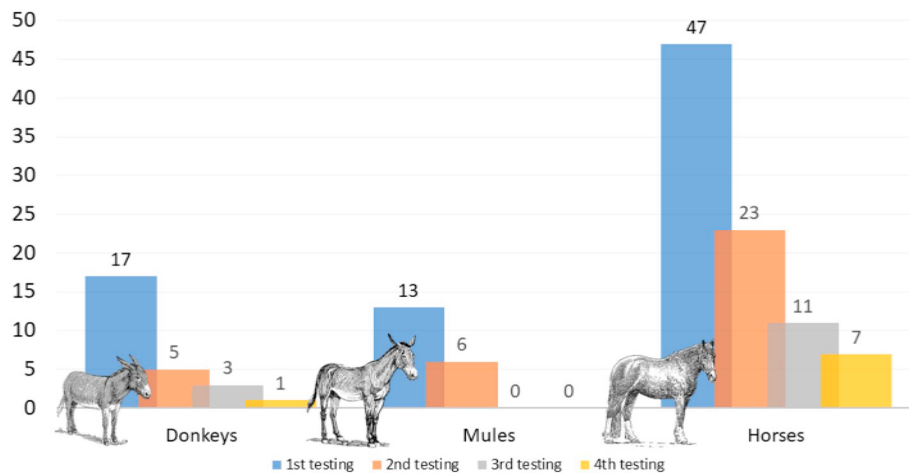


Fig. 1 Partially overlapping sample formation process.

violated the assumptions for regular parametric tests to report valid results.

2.1. Statistical analyses

In our study, we compare observations taken at different points in time, where the population membership changes over time but retains some common members. This approach is addressed in literature as the comparison between samples with both paired observations and independent observations (or partially overlapping samples). The use of paired or independent affine approaches, discarding the independent data or treating independent data as missing observations (REML repeated measures ANOVA with missing observations), is likely to be routinely implemented, when normality, sphericity and homoscedasticity assumptions are still met.

However, according to Derrick et al. (2017b), the assumptions of the partially overlapping samples *t*-test with Welch's degrees of freedom match the assumptions of Welch's test, considering those partially overlapping observations within a sample are independent of each other and are sampled from normally distributed populations. When normality assumptions have not been grossly violated, according to Rasch et al. (2011) Welch's test's power comes close to that of the *t*-test when the variances are homogeneous, and for unequal variances and skewness values < 3, it keeps the so called 20% robustness. By contrast, when there has been a gross violation of normality, as in our study, Lantz (2013) concluded that samples should be considered independent, as Kruskal-Wallis test is considerably less sensitive to the degree of sample normality and should therefore be the primary tool used to compare sample statistics, rather than ANOVA with repeated measures, Brown-Forsythe test or Welch's test.

All assumptions except for Shapiro Wilk Francia's normality tests were carried out using SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). Shapiro Wilk Francia's normality tests were carried out

with the .sfancia routine of StataCorp Stata version 14.2. The factors and variables in the model violated most of the common parametric assumptions, thus the decision to follow a nonparametric approach was made.

A Kruskal-Wallis H was performed to study the potentially existing differences between concentrations of the same factor when three or more groups existed within the same independent variable (all independent variables except for year) and a Mann-Whitney *U* test was used to compare differences between the two independent groups of the year variable (2016 and 2017) as reported in Table 2.

After conducting the Mann-Whitney *U* test for the year factor and the Kruskal-Wallis H for those variables with three or more groups (*k*), computation of the strength effect of the factors on the variables tested using *r* and partial eta squared (η^2), respectively, as measures of the strength of the factors the variables tested (Table 2). According to Fritz et al. (2012) *r* can be calculated as an effect size for the Mann-Whitney *U* test using the formula:

$$z/\sqrt{N}$$

z can be calculated by hand (Siegel and Castellan, 1988). The *z* value can be used to calculate an effect size, such as the *r* proposed by Cohen (1988); Cohen's guidelines for *r* are that a small effect is 0.1, a medium effect is 0.3, and a large effect is 0.5 (Coolican, 2009). Calculation of *r*, r^2 , or η^2 from these *z* values is possible because.

$$z/\sqrt{N} \text{ and } r^2 \text{ or } \eta^2 = z^2/N$$

These effect size estimates remain independent of sample size despite the presence of *N* in the formulas. This is because *z* is sensitive to sample size; dividing by a function of *N* removes the effect of sample size from the resultant effect size estimate (Coolican, 2009).

After Kruskal Wallis H test, we use partial eta² (η^2) value to numerically express that the effect for group differences accounts for the values of such differences plus their associated error variance, to then

Table 1
Normality assessment of SAA maximal concentration expressed in mg/L.

Factor	Type	Levels
Sample time point	Nominal	First, second third and fourth sampling.
Species	Nominal	Donkey, Mule, Horse
Month	Nominal	January, February, March, April, May, June, July, August, September, October, November, and December.
Season	Nominal	Autumn, Winter, Summer, and Spring.
Year	Ordinal	2016, 2017.
Clinical diagnose diagnosed	Nominal	Mouth Abscess, Foot Abscess, Anorexia, Septic Arthritis, Colic, Colitis, Impaction, Colitis + Foot Abscess, Dyspnoea, EHV-1 (Encephalomyelitis), Hepatitis, Umbilical Hernia, Hoof Wound + Hemorrhage, Radial Neuropathy, Esophagus Obstruction, Omphalophlebite, Facial Paralysis, Peritonitis, Wound, Pneumonia, Post-Surgical (Enucleation), Digestive Strongyles, Synovitis, Tendinitis, Tetanus, Traumatism, Ulcer + Oral Necrosis, Uveitis.

Table 2
Summary of the results of the Mann-Whitney U test and Kruskal-Wallis H test and the determinative coefficient (through r and partial eta squared (p^2), respectively SAA maximal concentration expressed in mg / L in working equids.

Factor	χ^2	dfn	Sig.	dfd	F	r^2 /Partial eta squared ^b	Interpretation	Signs
Sample time point	4.041	3	0.257	127	1.347	0.031 ^a	Small non significant effect	No effect can be detected
Species	14.845	2	0.001	130	7.423	0.102 ^a	Medium highly significant effect	Differences can be detected with Laboratory tests
Clinical diagnose	57.369	27	0.001	105	2.125	0.353 ^a	Medium to large highly significant effect	Differences can be sensed with naked eye, however laboratory analyses are needed to detect differences
Month	10.402	11	0.495	121	0.946	0.079 ^a	Small non significant effect	No effect can be detected
Year	14.628	1	0.001	131	14.628	0.317 ^b	Medium highly significant effect	We can sense differences but laboratory analyses are needed to detect differences
Season	2.102	3	0.551	129	0.701	0.016 ^a	Small non significant effect	No effect can be detected

^a After Mann-Whitney U test; ^bAfter Kruskal-Wallis H test.

turn it into a percentage. Through the use of partial eta square, we report an index of the strength of association between an independent variable and a dependent variable that excludes the variance produced by other variables (Brown, 2008). Because the Kruskal-Wallis test statistic is based on a single independent factor entered analysis and consequently, no other variable accounts for variance in the dependent variable, p^2 equals r^2 . This property is especially relevant when testing empirical variables among which there no possibility of overlapping, such as those in this study. Kruskal-Wallis H produces χ^2 values with k-1 degrees of freedom. We can transform χ^2 into an F value with k-1 numerator degrees of freedom (dfn) and N-k denominator degrees of freedom (dfd) using the expression $F(df_n, df_d) = \chi^2 / (k-1)$, modified from Murphy et al. (2014). From $F(df_n, df_d)$ we calculate partial eta squared (Lakens, 2013) from the following equation $p^2 = (F \cdot df_n) / (F \cdot df_n + df_d)$. p^2 can be benchmarked against Cohen (1988) criteria of small (0.01), medium (0.09), and large (0.25) effects as suggested in Richardson (2011).

In Cohen's terminology, a small effect size is one in which there is a real effect but which you can only see through careful study. By contrast, a 'large' effect size is an effect which is big enough, and/or consistent enough, that one may be able to see it 'with the naked eye'.

As all the variables have been previously reported to be non-normally distributed (Shapiro-Wilk Francia's tests ($P < .001$), an independent-sample median test was carried out to assess the differences in the median between categories (species and clinical diagnoses) within the same factor (Table 3).

Afterwards, we studied the pairwise comparisons for any dependent variables for which the Kruskal-Wallis test is significant aiming at assessing whether there are the differences between groups of the same factor affecting them using the Dunn's test (Tables 4 and 5) (Dinno, 2015; Morton, 2000). The Bonferroni correction compensates for the increase in the rejection of the existence of statistically significant differences between two or more groups by testing each hypothesis (comparison) at a significance level of α/m , where α is the desired overall alpha level, and m is the number of hypotheses.

Categorical regression (CATREG) was performed to the three independent factors (fixed effects) reporting significant differences between levels (species, year, and clinical diagnose diagnosed) and SAA maximal concentration expressed in mg / L as the dependent variable. Then stepwise linear regression to the data with the resulted quantifications was applied and is presented in the summary results with the significant variables in Tables 7 and 8.

All nonparametric tests were carried out using the independent samples package from the non-parametrical task of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). Categorical regression (CATREG) on the data was used to describe how our variables linearly depended on the predictors (factors) considered. The resulting regression equation can be used to describe the relationship of Serum Amyloid A concentrations with any combination of the factors reported to be statistically significant at CATREG analysis. We performed a categorical regression with the Optimal Scaling procedure from the Regression task from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016).

3 Results

Normality (Shapiro Wilk's Francia W, $P < .001$), homoscedasticity (Levene's test for equality of error variance, $P < .05$) and sphericity (Mauchly's W = 0.001), $\chi^2(20) = 1488.661$, $P < .05$) assumptions for SAA maximal approximate concentration across the different levels for almost all the factors studies were violated. Almost all significant univariate outliers fell within the range of values for SAA maximal concentration found in literature for the species considered in the study (Nolen-Walston, 2015). Hence, our decision to retain them in the analysis.

For year, it can be concluded that SAA concentration in 2016 group was highly statistically significantly higher than 2017 (U = 1266,

Table 3
Summary of the results of the independent sample median test for the effects of species and clinical diagnose on SAA maximal concentration in working equids.

Variable	Descriptive statistics	Level	> Median	≤ Median
Species	N = 130	Donkey	9	15
	Median = 447.00	Mule	5	14
	Chi-Square = 8.349	Horse	51	36
	df = 2			
Clinical diagnosis	Asymp. Sig. = 0.015			
	N = 130	Mouth abscess	0	3
	Median = 447.00	Foot abscess	0	2
	Chi-Square = 44.750	Anorexia	1	0
	df = 27	Septic arthritis	0	1
	Asymp. Sig. = 0.017	Colic	0	1
		Colitis	32	24
		Impaction	3	0
		Colitis + Foot Abscess	1	0
		Dyspnea	0	4
		EHV-1 (Encephalomyelitis)	0	4
		Hepatitis	0	1
		Umbilical hernia	1	0
		Hoof Wound + Hemorrhage	1	0
		Radial neuropathy	0	1
		Esophagus obstruction	1	1
		Omphalophlebitis	2	1
		Facial paralysis	4	0
		Peritonitis	2	2
		Wound	0	4
		Pneumonia	8	11
		Post-Surgical (Enucleation)	1	0
		Digestive strongyles	4	1
		Synovitis	0	1
		Tendinitis	0	1
		Tetanus	0	1
		Traumatism	1	0
		Ulcer + Oral Necrosis	3	0
		Uveitis	0	1

$P < .001$). The Mann-Whitney U test was used to compare differences between the two independent groups of the year variable (2016 and 2017).

The study reports the results from Kruskal Wallis H for all the variables and concentrations considered in the study and r and partial eta squared (p^2) as a measure of the strength of the factors the variables tested (Table 2). Table 3 shows the results for the independent-sample median test addressing the differences in the median between the categories considered within the factor of species and clinical diagnoses. Tables 5 and 6 show the results for Dunn tests' pairwise comparison between the different concentrations of the factors and variables.

CATREG was performed on the three independent factors reporting significant differences between concentrations (species, year, and clinical diagnosis) and SAA maximal concentration expressed in mg / L as the dependent variable. A stepwise linear regression to the data with the resulted quantifications was then applied; the data is presented in the summary results with the significant variables in Table 6. The standardized coefficients (β) are listed in Table 6 as well.

CATREG reported all of the independent variables except for month and season to be significant for all the variables tested. After Categorical regression, including significant factors on SAA, the Model explained 33 % of the variability in SAA (Table 7).

We found 10.2 % of the total variation in the concentration of SAA could be explained by the species factor. Then a 27 % of this 10.2 effect holds a linear relationship with SAA concentrations with donkeys scoring the lowest linear relationship, mules presented double the linear relationship presented by donkeys and horses three times the relationship presented by donkeys. That is, 10.2 % of the SAA concentration in a certain animal could be attributed to its species (or it being a hybrid), with values for SAA concentration progressively increasing as the species that the animal belongs to is closer to the horse. Hence, differences can be detected with laboratory tests, such as those

reported by Henze et al. (2011).

The 43.8 % linear relationship between the clinical diagnose factor and SAA concentration with clinical diagnose explaining 35.3 % of total variance in SAA concentrations indicated that differences can be sensed with naked eye, however laboratory analyses are needed to detect differences among clinical diagnoses. The standardized solutions for the predictive regression equations can be found in Table 8.

When we studied the differences between species, a highly statistically significant difference was found when comparing SAA concentrations of mules to horses ($P < .001$). Similarly, a statistically significant difference ($P < .05$) was found between donkeys and horses, but not donkeys to mules ($P > .05$) using the Dunn's pairwise comparison Test. The results for the median t for independent samples suggested most of the cases reporting a value for SAA concentration over the median belonged to horses (58.62 %), these were followed by donkeys (37.5 %) and mules (26.31 %). This trend inverted with mules, reporting the highest frequency for SAA concentrations below the median (73.69 %), followed by donkeys (62.5 %), and horses (41.38 %). Comparing these results to those reported by Kruskal Wallis H test and Dunn's test, it could state that horses more frequently displayed statistically significantly values for SAA concentration over 447 mg/L than donkeys and mules. By contrast, the differences between donkeys and mules were not significant from what could be inferred, both equally reported values under or equal to 447 mg/L.

Fig. 2 shows there was an unbalanced distribution of cases among clinical diagnosis during the two-year study. That is, there were clinical diagnosis groups with an incidence of only one case while there were other cases, such as colitis with 54. Given the data was unbalanced, this study did not find significant differences between the clinical diagnosis groups of anorexia, hepatitis, umbilical hernia, hoof wound and consequent hemorrhage, esophagus obstruction, peritonitis, post-surgical (enucleation), tetanus and traumatism.

When comparing clinical diagnosis, certain conditions always

Table 4
Summary of the significant ($P < .05$) species/hybrid pairwise comparison obtained after Dunn's test for SAA maximal concentration expressed in mg / L in working equids.

Clinical diagnosis pairwise comparison	Contrast statistic	SE	Contrast statistic deviation	Sig.
Colic vs digestive strongyles	-86.40	41.24	-2.10	0.04
Colic vs Facial Paralysis	-101.00	42.09	-2.40	0.02
Colic vs Impaction	-106.33	43.47	-2.45	0.01
Colic vs Ulcer + Oral Necrosis	-100.67	43.47	-2.32	0.02
Dyspnea vs Colitis	57.68	19.48	2.96	0.00
Dyspnea vs Colitis + Foot Abscess	92.75	42.09	2.20	0.03
Dyspnea vs Digestive Strongyles	-80.15	25.25	-3.17	0.00
Dyspnea vs Facial Paralysis	-94.75	26.62	-3.56	0.00
Dyspnea vs Impaction	100.08	28.75	3.48	0.00
Dyspnea vs Omphalophlebitis	-70.25	28.75	-2.44	0.02
Dyspnea vs Pneumonia	-49.41	20.71	-2.39	0.02
Dyspnea vs Ulcer + Oral Necrosis	-94.42	28.75	-3.28	0.00
EHV-1 vs Colitis	55.30	19.48	2.84	0.01
EHV-1 vs Colitis + Foot Abscess	90.38	42.09	2.15	0.03
EHV-1 vs Digestive Strongyles	-77.78	25.25	-3.08	0.00
EHV-1 vs Facial Paralysis	-92.38	26.62	-3.47	0.00
EHV-1 vs Impaction	97.71	28.75	3.40	0.00
EHV-1 vs Omphalophlebitis	-67.88	28.75	-2.36	0.02
EHV-1 vs Pneumonia	-47.03	20.71	-2.27	0.02
EHV-1 vs Ulcer + Oral Necrosis	-92.04	28.75	-3.20	0.00
Foot Abscess vs Digestive Strongyles	-71.40	31.49	-2.27	0.02
Foot Abscess vs Facial Paralysis	-86.00	32.60	-2.64	0.01
Foot Abscess vs Impaction	-91.33	34.36	-2.66	0.01
Foot Abscess vs Ulcer + Oral Necrosis	-85.67	34.36	-2.49	0.01
Mouth Abscess vs Digestive Strongyles	-57.23	27.49	-2.08	0.04
Mouth Abscess vs Facial Paralysis	-71.83	28.75	-2.50	0.01
Mouth Abscess vs Impaction	-77.17	30.74	-2.51	0.01
Mouth Abscess vs Ulcer + Oral Necrosis	-71.50	30.74	-2.33	0.02
Pneumonia vs Facial Paralysis	45.34	20.71	2.19	0.03
Pneumonia vs Impaction	50.68	23.39	2.17	0.03
Pneumonia vs Ulcer + Oral Necrosis	-45.01	23.39	-1.93	0.05
Radial Neuropathy vs Digestive Strongyles	-86.40	41.24	-2.10	0.04
Radial Neuropathy vs Facial Paralysis	-101.00	42.09	-2.40	0.02
Radial Neuropathy vs Impaction	106.33	43.47	2.45	0.01
Radial Neuropathy vs Ulcer + Oral Necrosis	-100.67	43.47	-2.32	0.02
Septic Arthritis vs Facial Paralysis	-92.50	42.09	-2.20	0.03
Septic Arthritis vs Impaction	-97.83	43.47	-2.25	0.02
Septic Arthritis vs Ulcer + Oral Necrosis	-92.17	43.47	-2.12	0.03
Synovitis vs Digestive Strongyles	86.40	41.24	2.10	0.04
Synovitis vs Facial Paralysis	101.00	42.09	2.40	0.02
Synovitis vs Impaction	106.33	43.47	2.45	0.01
Synovitis vs Ulcer + Oral Necrosis	-100.67	43.47	-2.32	0.02
Tendinitis vs Digestive Strongyles	86.40	41.24	2.10	0.04
Tendinitis vs Facial Paralysis	101.00	42.09	2.40	0.02
Tendinitis vs Impaction	106.33	43.47	2.45	0.01
Tendinitis vs Ulcer + Oral Necrosis	-100.67	43.47	-2.32	0.02
Uveitis vs Digestive Strongyles	86.40	41.24	2.10	0.04
Uveitis vs Facial Paralysis	101.00	42.09	2.40	0.02
Uveitis vs Impaction	106.33	43.47	2.45	0.01

reported values over 447 mg/L (mouth abscess, foot abscess, septic arthritis, colic, dyspnea, EHV-1, radial neuralgia, wound, synovitis, tendinitis and uveitis). However, out of this group of clinical diagnoses

Table 5
Summary of the significant ($P < .05$) diagnosed clinical diagnose pairwise comparison obtained after Dunn's test for SAA maximal concentration expressed in mg / L in working equids.

Species/Hybrid pairwise comparison	Contrast Statistic	SE	Contrast statistic deviation	Significance
Donkey vs Mule	15.935	11.559	1.379	0.168
Mule vs Horse	-34.140	9.532	-3.582	0.001
Horse vs Donkey	-18.205	8.679	-2.098	0.036

scoring above the median, only mouth abscess, foot abscess, dyspnea, EHV-1 and wound were presented in more than two occasions. Anyway, conditions being presented only once were still considered as they represent the distribution of cases found in the population of working equids in the African continent (Getachew et al., 2016; Getachew et al., 2014; Kiros et al., 2016; Pritchard et al., 2005; Stringer et al., 2017). Following this group of reduced incidence clinical diagnosis, the clinical diagnosis reporting the highest frequency of cases below or equal to 447 mg/L (median) was pneumonia (57.89), followed by colitis (42.86), omphalophlebite (33.33) and digestive strongyles (20). The inverse trend in ascending order ranking those clinical diagnosis accounting for the highest frequencies of cases reporting values over 447 mg/L were colitis simultaneous to foot abscess (bearing in mind it was only presented once), facial paralysis (four times), and ulcer simultaneous to oral necrosis (three times) (100), followed by digestive strongyles (80 of 5 cases), omphalophlebite (66.67 of 3 cases), colitis (57.14 of 56 cases) and pneumonia (42.11 of 19 cases), as it can be inferred for the position, such clinical diagnosis reporting values over the median report a higher mean ranks. Table 5 shows those pairs of clinical diagnosis for which SAA concentrations were significantly different. The rest of differences are unreported as they were statistically non-significant.

4 Discussion

Given SAA concentrations short 24-h half-life, decreases rapidly occur with a positive response to treatment, enabling the monitorization of treatment effectiveness and increasing the predictability of treatment success. (Belgrave et al., 2013). This clinical context supports our non-significant results for differences from the one sample time point to the following ones, especially when treatment has been applied as normal concentrations in mules, donkeys likewise horses are undetectable and acutely increase to later decrease during acute inflammatory processes.

SAA has been reported to be more sensitive to the indication of inflammation and tissue damage, compared to that of fibrinogen and leucocyte counts (Jacobsen and Andersen, 2007). While the precise roles of SAA are still under investigation (Jacobsen and Andersen, 2007), current findings have shown SAA to enhance as well as inhibit leucocyte functions (Badolato et al., 2000; Gatt et al., 1998), influence synthesis of inflammatory mediators (Malle et al., 1997), affect lipid transportation to inflamed tissue (Gonnerman et al., 2009; Lindhorst et al., 1997) and induce enzymatic activity associated with degradation of the extracellular matrix (Vallon et al., 2001). Piovesana et al. (2015) reported SAA in donkey milk, which is found in great volumes in milk, is available but no references for application in disease diagnosis in mules and donkeys apart from it being suggested as a possible marker of mastitis in other species such as cows (Kovačević-Filipović et al., 2012).

Henze et al. (2011), while studying genetic differences in the serum proteome strongly reported the existence of a free cysteine residue also in the post-translational modifications protein transthyretin of horses, donkeys and mules, which may be important in the endogenous extracellular antioxidant defense system and oxidative stress of all three species. According to these authors, results probably indicate that the

Table 6
Model summary of stepwise linear regression with transformed variable for SAA maximal concentration expressed in mg / L in working equids.

Variable	R Square	Adjusted R Square	Significance	Apparent standard error
SAA maximal concentration expressed in mg / L	0.575	0.330	0.029	0.670

proteome of mules reveals a higher similarity to donkeys than to horses and may indicate an endogenous antioxidant defense system of horses, donkeys and mules comparable with humans and rats. However, due to the distinct differences in protein patterns of the three species, further analyses are needed to evaluate post-translational modifications of the whole proteomes.

With the aid of SAA, the new equations can be utilized to describe the found association between clinically diagnosed diseases and the SAA response across different species (Table 8). Donkeys and mules present a greater challenge for promptly identifying disease due to their dullness, depression, and even anorexia, as they show these signs only late in the course of the disease (Matthews et al., 2005). Our values match those reported in literature by (Nolen-Walston, 2015) for horses, as patients with systemic inflammation had significantly higher SAA (mean, 1583 mg/L; range, 688–4000 mg/L) than patients with local or no inflammation, which had mean SAA concentrations of 343 mg/L (range, 37–1609 mg/L) and 5.6 mg/L (range, 1.8–14.5 mg/L) respectively.

SAA has been especially useful for diagnosing respiratory diseases in horses (Viner et al., 2017). We know both mules and donkeys are susceptible for respiratory infection such as pneumonia and equine influenza (Pusterla et al., 2003). However, mules and donkeys may hide clinical signs of respiratory infection including but not limited to nasal discharge, cough, or evaluated temperature (Viner et al., 2017). Although, one study found clinical signs such as nasal discharge and elevated temperatures in a population of mules who were later diagnosed with strangles. Elevated concentrations of SAA have been observed in horses with clinical signs of pneumonia as well as horses infected with equine influenza virus (Jacobsen and Andersen, 2007). This finding supports our results as almost 50% of the cases in which pneumonia had been diagnosed reported values over the median (> 447 mg/L) while the rest of respiratory conditions reported values below the median in all cases. Research indicated SAA concentrations were a more sensitive marker for acute infection of equine influenza than nasal swabs as verified by clinical signs and seroconversion (Hulten et al., 1999). Serum concentrations were elevated for extended periods in horses with severe influenza, or those that contracted a secondary bacterial infection, and remained elevated for extended periods in comparison to those with a milder infection (Hulten et al., 1999). This information indicates that SAA measurements may be used to monitor the recovery of, and establishment of individual convalescent periods, animals suffering from equine influenza (Jacobsen and Andersen, 2007). There is a call for more research regarding the evolution of SAA concentration over time or response rates and usefulness of SAA in the diagnosis and monitoring of *Rhodococcus equi* pneumonia (Jacobsen and Andersen, 2007).

Equine herpesvirus 1 (EHV-1) has a major impact on reproductive capacity of mares and jennies. EHV-1 is an alpha herpes viral pathogen that induces abortions in pregnancy in addition to early neonatal death

in foals, respiratory disease in young equids, and myeloencephalopathy leading to significant financial losses to the owner (Pusterla et al., 2003; Soydal Ataseven et al., 2010). The virus can become latent in the infected animal's lymphoid tissues, particularly in the areas training the respiratory tract (Purewal et al., 1992). In the latent state, EHV-1 remains dormant, when reactivated the viruses propagates inevitably causing cell lysis and spreading throughout the system. In our population under study most, working equids being with EHV-1 exhibited signs of an upper respiratory infection. Respiratory infections can decrease the efficiency of these animals' ability to perform work. AHV-3 (type-3), considered the progenitor of EHV-1, is a host adapted virus seen in donkeys which may induce mild rhinitis or show no clinical signs of the virus (Barrandeguy and Carossino, 2018). Our results agree and are in the range of those reported by Nolen-Walston (2015) for horses, as those clinical conditions of a local nature presented mean SAA concentrations of 343 mg/L (range, 37–1609 mg/L) which are quite close to those reported in our study with medians of ≤ 447 mg/L. The pathogenic impact of AHV still remains unclear, with no available control or preventative measures (Barrandeguy and Carossino, 2018); even though there is much to be studied, SAA could serve as an initial indicator of virial infection and amplify the response rate (SAA concentration over time) and treatment given. Very few studies have suggested the existence of interspecific variation such as that reported by our study. Vissani et al. (2016) studied the susceptibility of equid herpesvirus 3 field isolates to antiviral compounds. Post-vaccination systemic inflammatory response (SIR) characterized by significantly increased SAA, but the intensity of SIR was not associated with a higher virus-neutralizing (VN) antibody titers for EHV-1. Interestingly, the immune response differed between groups, with higher antibody production in mules, but no significant response detected for SAA concentrations. This contrast our results, however, the smaller sample in the already mentioned study may have underrated the species effect. Studies indicate SAA has a prominent, more rapid, response to EHV-1 in pregnant mares as well as a foal's than fibrinogen (Hulten et al., 1999). Utilizing the SAA response to EHV-1 can serve as an important observation in view of serious consequences to the spread of this infectious pathogen among other animals (Vissani et al., 2016). With frequent and routine SAA screening, it is possible that SAA could provide early warning and lead to a quicker recognition of infection (Pepys et al., 1989).

Strangles, for example, is an infection caused by *Streptococcus equi* commonly associated with fever, lethargy anorexia, nasal discharge, and abscessation of lymph glands in the head and neck (Ijaz et al., 2010). This infection can last for months or years within herds and is highly contagious. While there is ample research on strangles in horses, little is known of the prevalence of *Streptococcus equi* or its effect on serum protein in mules. Ijaz et al. (2010) investigated strangles in mules. The study tested serum albumin, serum globulin and fibrinogen from 250 positively diagnoses mules in Pakistan (Ijaz et al., 2010). The

Table 7
Standardized Coefficients and significance of CATREG model for SAA maximal concentration expressed in mg / L in working equids.

Variable	SAA maximal concentration expressed in mg / L				
Factor	Standardized Coefficients (β)	Bootstrap (1000) estimation of standard error	df	F	Significance
Species	0.270	0.123	2.000	4.837	0.010
Clinical diagnosis	0.438	0.080	22.000	29.654	0.000
Year	−0.148	0.125	1.000	1.404	0.239

Table 8
Regression equations for SAA maximal concentration expressed in mg / L in working equids.

General model regression equation			Legend
$Z'y_{SAA\text{Admh}} = \beta_{\text{species}} * Z_{\text{species}} + \beta_{\text{clinical diagnosis}} * Z_{\text{clinical diagnosis}}$			$Z'y_{SAA\text{Admh}}$ = Z score for SAA maximal concentration expressed in mg / L β = standardized coefficient for each of the factors appearing in the subindex. Z = Z score for each of the factors appearing in the subindex.
Specific regression equations			Legend
n = 13	Donkeys	$Z'y_{SAA\text{Ad}} = 0.270 + 0.438(Z_{\text{clinical diagnosis}}) \pm 0.670$	$Z'y_{SAA\text{Ad}}$ = Z score for SAA maximal concentration in donkeys. $\beta_{\text{clinical diagnosis}} Z_{\text{clinical diagnosis}} = 0.438(Z_{\text{clinical diagnosis}})$
n = 17	Mules	$Z'y_{SAA\text{Am}} = 0.540 + 0.438(Z_{\text{clinical diagnosis}}) \pm 0.670$	$Z'y_{SAA\text{Am}}$ = Z score for SAA maximal concentration in mules. $\beta_{\text{clinical diagnosis}} Z_{\text{clinical diagnosis}} = 0.438(Z_{\text{clinical diagnosis}})$
n = 47	Horses	$Z'y_{SAA\text{Ah}} = 0.810 + 0.438(Z_{\text{clinical diagnosis}}) \pm 0.670$	$Z'y_{SAA\text{Ah}}$ = Z score for SAA maximal concentration in horses. $\beta_{\text{clinical diagnosis}} Z_{\text{clinical diagnosis}} = 0.438(Z_{\text{clinical diagnosis}})$

Year^(a) resulted statistically non-significant (P > .05) in CATREG analyses.

study concluded that total serum proteins, in combination with a decreased serum albumin level, were vital indicators of infection (Ijaz et al., 2010). A study on *Streptococcus equi* serovar *equi* *S. equi*) in mules, suggested elevated concentrations of serum proteins, serum globulin and fibrinogen with the associated decrease in serum albumin were significant markers of infection (Mendoza et al., 2018). One such factor that was found significant among mules, exhibiting signs of infection, was the age of the animal. The incidence of strangles was highest in mules up to two years of age, providing an example of one such factor that may affect the concentrations of SAA seen in this study when comparing species (Ijaz et al., 2010).

The etiology of acute colitis cases differs from individual to individual and specific diagnosis is often not found (Cohen and Divers, 1998a; Feary and Hassel, 2006). Among the etiologies for acute colitis

at the Fondouk include dietary change, antibiotic use, intoxication, parasitism, idiopathic and grain overload as described in literature (Atherton et al., 2009). Some of these animals will have shown a proliferation of pathogenic bacterial organisms in the colon, secondary to the primary insult, specifically salmonella and clostridia, and these animals tend to have the highest SAA concentrations and the worst outcomes (Cohen and Divers, 1998b).

Circulating concentrations of SAA were significantly higher at admission in horses with colic attributable to conditions having a primary inflammatory cause (eg, enteritis, colitis, peritonitis, or abdominal abscesses) and were higher in horses that failed to survive the episode of colic, compared with concentrations in horses that survived (Vandenplas et al., 2005), as it has been reported by our results, matching those by Nolen-Walston (2015) regarding systemic conditions

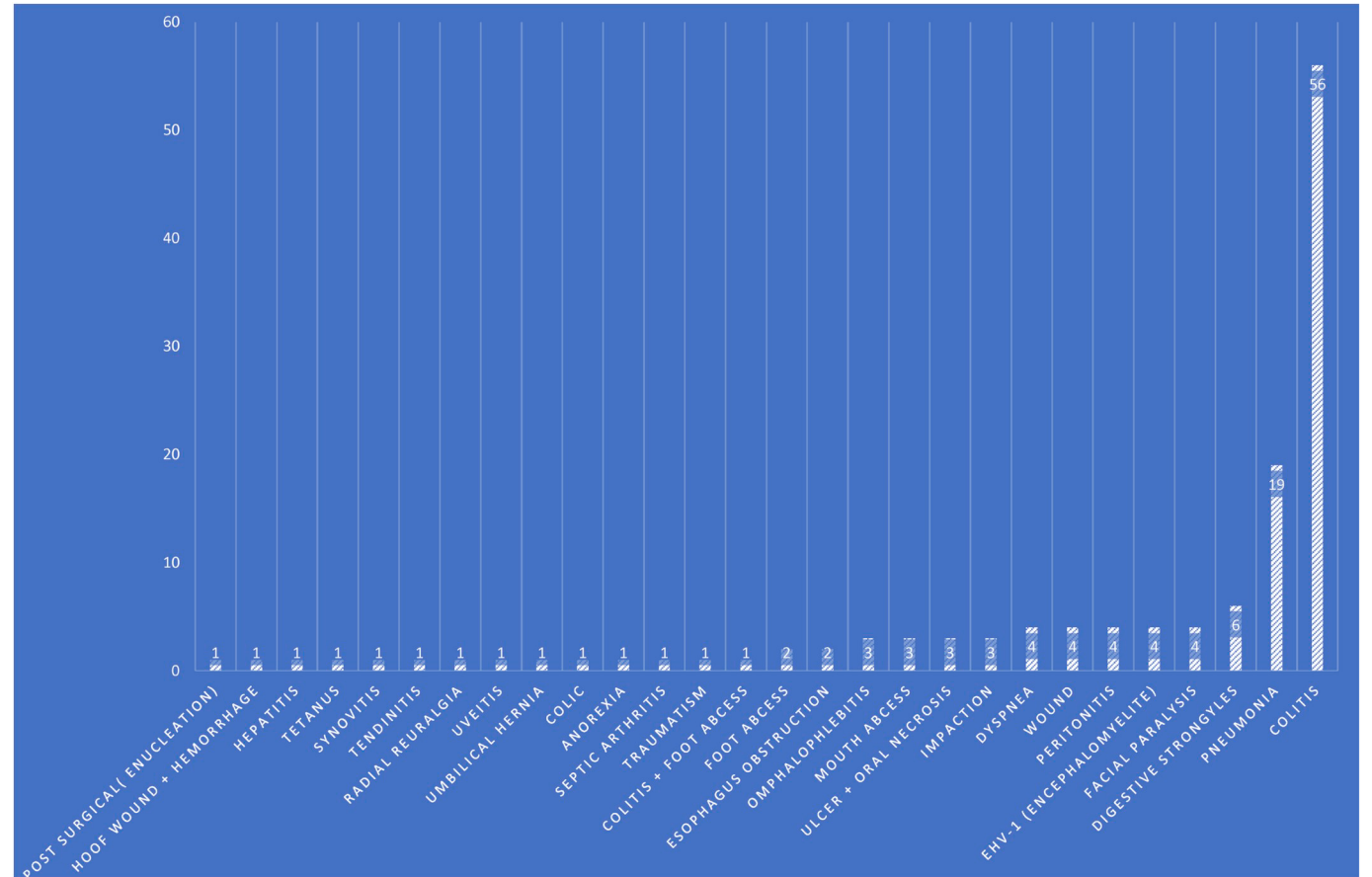


Fig 2 Case frequency sorted per clinical diagnosis.

(mean, 1583 mg/L; range, 688–4000 mg/L) for horses, given the fact that despite the health issues underlying colic cases may have a local origin, bacteria and endotoxins may end up entering the bloodstream, leading to further systemic effects. Studies have shown that SAA can assist in the identification of colitis. A study in mice affected with colitis found that intestinal epithelial SAA concentrations displayed bactericidal properties indicating altered SAA expression may be involved in the disease (Eckhardt et al., 2010). Mice genes were altered to inactive two major acute phase isoforms (SAA1 and SAA2) then challenged with DSS in drinking water to induce colitis (Malle et al., 1997). The study also tested if over-expression of SAA in cultured enterocytes would reduce co-cultured *E. coli* viability. The study found that overexpression of SAA 1 and SAA 2 had a strong reduction in the viability of co-cultured *E. coli*; concluding that intestinal epithelial SAA may play a protective role in colitis cases (Eckhardt et al., 2010). This study correlates with the study conducted by Eckhardt and associates, in which the data presented elevated SAA concentrations in all equids, horses, donkeys, and mules diagnosed with colitis suggesting SAA can be used to detect colitis in future cases.

It is also known that SAA is a valuable tool for measuring inflammation associated with lameness such as hoof abscesses (Jacobsen and Andersen, 2007). This study found that abscesses were present in multiple cases and their SAA concentration were always over the median (> 447 mg/L). However, other hoof wounds that may not be related to systemic inflammatory processes reported values below the median in all cases. A lame donkey with a swollen leg, for example, may have acute life-threatening cellulitis, or may have simple edema secondary to trauma. These two clinical diagnoses will require totally different treatment regimens and the SAA value is extremely valuable in aiding the veterinarian establish the correct diagnosis (Jacobsen and Andersen, 2007). Working equids are subject to continuous chronic fatigue of limbs due to the work demands placed on them increasing the energy demand required to continue to function at the capacity expected of them (Viner et al., 2017). SAA has the potential to serve as a useful measurement tool for a variety of different infections and diseases.

Breed factors have been shown to affect parameters such as insulin concentrations, body condition scores, and concentrations of SAA (Eckhardt et al., 2010). Similarly, a simultaneous positive correlation between BCS and insulin concentration has been reported together with an increase in SAA concentrations (Eckhardt et al., 2010). There is a paucity of data regarding baseline hormone ranges in donkeys, however, current studies comparing donkey hormone values, such as adrenocorticotrophic hormone (ACTH) insulin and cortisol, to those of horses are even more infrequent. On the other hand, it is important to note metabolic parameters in the donkey differ with gender and physiological state (Broster et al., 2009; Suagee et al., 2013). Jennies that are healthy and nonpregnant have been shown to have lower glucose and higher concentrations of triglycerides than jacks that is likely related to sex hormones and fat distribution between genders (Dugat et al., 2010). Dugat et al. (2010) and associates, compared insulin values as well as ACTH, triglycerides, and cortisol of donkeys to the associated hormone concentrations of horses; the study found that donkeys, in various classifications (normal nonpregnant, pregnant, stallions, and yearlings) exhibited insulin concentrations below the normal range of horses (Suagee et al., 2013). Suagee et al. (2013) suggests an increase in BCS and insulin results in an increase in SAA (Eckhardt et al., 2010). In donkeys, we know that insulin is typically lower than concentrations found in horses which may lead to or explain the lower level of SAA found in donkeys and mules (Eckhardt et al., 2010). Additionally, the study found that there were differences between horse and donkey ACTH and triglyceride values (Suagee et al., 2013) further suggesting that there are biological differences exhibited between horses, donkeys, and the hybrid mule. Studies comparing insulin values of mules and horses has yet to be conducted but we do know other biochemical and hematocrit values can stand alone when

compared to horses and donkeys (McLean et al., 2016).

Further research is needed to determine the mules' biological tolerance to pathogens and the body's inflammatory response. Such studies could provide information pertaining to SAA concentrations in mules, insulin, BCS, and other cytokine markers when compared to horses and donkeys and if a difference is continued to be found this could suggest the inflammatory response is less in mules which could be part of hybrid vigor.

Though there is little research on SAA in mules and donkeys, SAA has been identified as a useful marker for inflammation due to its rapid response rate (SAA concentration over time) in which concentrations increase up to 5000–10,000 mg/L within a few hours in horses (Viner et al., 2017). With the novelty of SAA, current research seeks to better understand its functionality and applicability to infectious and non-infectious diseases. Additional studies have reported high plasma SAA concentrations in equine due to bacterial and viral infections including infectious arthritis, strangles, pneumonia, sepsis, equine herpes, and influenza virus (Broster et al., 2009; Ijaz et al., 2010; Jacobsen and Andersen, 2007).

Collective research indicates observation and monitoring of the SAA concentration over time is a useful tool to assist in the diagnosis and treatment of various clinical cases seen in horses. There is a scarcity of research on SAA concentrations in mules. This study indicates that there may be a difference between the inflammatory response rate of mules versus horses. However, population numbers were low, and the different nature of the clinical diagnosis encountered may have influenced the results. The data provided indicates a call for additional research to determine the causes for these significant differences. Having a robust understanding of the role SAA plays in hybrids, such as the mule, in comparison to horses and donkeys will provide pertinent information to the veterinary field in terms of clinical treatment and response rates in all species of equids.

5 Conclusions

The results of this study begin to establish a better understanding of serum amyloid A concentrations in working equids of Morocco. Preliminary data indicated a difference in SAA response in mules versus horses or donkeys, suggesting that potential variables specific to a hybrid species may impact the inflammatory response rate or SAA concentration over time. Further research is necessary to further develop an understanding of SAA concentrations in horses, mules, and donkeys. This study serves as a foundation to better understanding the relationship SAA has in sick equids in Morocco with the potential to improve working equid welfare. Additional research on clinically healthy horses, mules, and donkeys can provide supplemental information to this research and allow for comparison between two clinically different samples to better identify SAA as a reliable marker in equids.

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