Comparative Immunology, Microbiology and Infectious Diseases 64 (2019) 25–30

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

# Serum haptoglobin response in red deer naturally infected with tuberculosis

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ARTICLE INFO

*Keywords:*
Acute phase proteins
*Cervus elaphus*
Haptoglobin
*Mycobacterium tuberculosis*complex
Comparative skin test

ABSTRACT

The analysis of haptoglobin (Hp) serum concentration is a very sensitive, but non-specific, indicator of inflammation or infection. Methods to accurately diagnose infection in vivo in wildlife are usually constrained by low sensitivity due to the effects of stress on individual immune response and the challenging logistics of performing tests in the wild. Firstly, we sought to determine serum Hp concentration in red deer (*Cervus elaphus*) naturally infected with bovine tuberculosis (TB). Secondly, we assessed the complementary diagnostic value of serum Hp levels in conjunction with the cervical comparative skin test (CCT) performed in a subsample ($n$ = 33). Serum Hp concentrations were significantly higher in TB-infected individuals (based on the presence of macroscopic lesions confirmed by culture) compared to those uninfected. In addition, serum Hp significantly changed with the type of animal handling, with captured and handled animals showing higher levels of Hp than hunted animals. Four out of 6 TB positive individuals that tested negative to the CCT (false negatives) showed Hp levels higher than the 95th percentile of healthy animals. These findings indicate that an acute phase response develops in animals with TB. In this paper, we demonstrate for the first time that an acute phase protein can provide a complementary assessment for specific diagnosis tests in wild species.

## 1. Introduction

Positive acute phase proteins (APPs) are plasma proteins that increase in concentration following tissue damage caused by inflammation, infection or trauma, even in subacute and chronic conditions [1]. They are very sensitive biomarkers for nearly any disturbance to the health of an organism. The acute phase response precedes antibody synthesis [2] and in field conditions, increased serum APPs are detected in sick animals before specific antibodies. Therefore, although less specific than serology, APPs are an earlier and more sensitive marker of disease [3].

Tuberculosis is a low-immunity induced and chronic infectious disease, which affects a broad range of hosts [4,5]. Because of mycobacteria-induced destruction of host tissues, the predominant expression of tuberculosis is granulomatous lesions of variable location, diameter and consistency, which may become encapsulated. Bovine tuberculosis (TB) in cattle is a major economic problem in Spain [6]. The increase in the population of large game mammals in recent decades has contributed to the maintenance of TB in the wild, which has likely impeded eradication schemes in domestic livestock [7,8].

In vivo diagnosis of TB infection in wild red deer *Cervus elaphus* by cervical comparative skin test (CCT) can be more constrained than in farmed red deer [9] due to the lack of sensitivity of the test under certain circumstances (such as stress) or unsuitable logistics in the wild. This could make CCT an ineffective test for controlling TB in the field. APPs may have the potential to complement specific diagnostic tests for infectious diseases in wild animals, which could be very valuable for disease management in wildlife, especially during wildlife translocations in which removing infectious individuals is crucial to control pathogen transmission and spread between populations in wildlife reservoirs [10].

Haptoglobin (Hp) has been applied previously to assess potential stressors in a few wildlife populations, suggesting that Hp concentration may be used as a biomedical indicator for health control: in *Lutra Canadensis* [11]; *Pusa hispida* [12]; *Eumetopias jubatus* and *Phoca vitulina* [13]. In ruminants, Hp is considered a major APP and until now, studies have been performed in *Capra ibex* [14] and farmed *C. elaphus* [15,16]. However, to our knowledge, the study of APPs in relation to infections has never been carried out in wild deer, and little is known about their APP response or its value as a potential biomarker for monitoring

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https://doi.org/10.1016/j.cimid.2019.01.021
Received 2 February 2018; Received in revised form 21 January 2019; Accepted 23 January 2019

specific diseases, animal health and welfare, especially in field conditions. Cross et al. [15] stated that the potential usefulness of Hp as an acute phase marker in deer lies in the fact that in healthy individuals, levels are low or absent. In this context, we aimed to study the response of Hp levels in relation to TB infection in wild red deer in southern Spain.

## 2. Materials and methods

### 2.1. Study animals and sampling

Data were collected from a cross-sectional national survey of TB infection in adult red deer hinds ($n = 88$) across hunting estates in south-central Spain [17]. Fifty-five of these animals were shot by hunters during the regular hunting season (October - January) and 33 were captured by keepers. Blood was collected from each deer by puncture of the heart or jugular vein (before TB test) and was transferred into sterile tubes without anticoagulant immediately after extraction. All samples were maintained at 4 °C during transport and centrifuged for about 4 h after sampling. Serum was obtained by centrifugation (at 2000 *g* for 10 min) and frozen at −20 °C until analysis.

### 2.2. Diagnosis of tuberculosis infection

Hunted red deer included in this study were subjected to detailed field necropsy and gross pathology studies. Lymph nodes were dissected, sectioned serially and systematically examined for gross lesions. The necropsy examination included detailed macroscopic inspection of lymph nodes and abdominal and thoracic viscera. This examination routinely included the parotid, lateral and medial retropharyngeal and submandibular lymph nodes of the head. The tracheobronchial and mediastinal lymph nodes and lungs were examined within the thorax. Within the abdomen, the hepatic, mesenteric and ileocaecal lymph nodes, ileocaecal valve, liver, kidneys and spleen were examined. Any gross lesions in other locations were also recorded. Lymph nodes were dissected and sectioned serially. Tonsils were routinely sampled for culture, but were not sectioned. Any gross tonsillar lesions were recorded. A detailed description of TB-like lesions can be seen in Martín-Hernando et al. 2010. Basically, lesions varied from firm or hard white, grey, or yellow nodules with a yellow, caseous, necrotic centre that was dry and solid to thin-walled suppurative abscesses. Animals without these lesions were classified as gross lesions negative [17,18]. TB diagnosis was based on the presence of macroscopic lesions in lymph nodes and abdominal and thoracic organs, and was confirmed by positive culture of mycobacteria and subsequent spoligotyping [19]. Briefly, two grams of sample were homogenized with 35 mL of a solution (0.75%) of hexadecilpiridinium and left decontaminating for 18 h. Each of four tubes of Coletsos medium (Bio-Mérieux, Marcy-l'Étoile, France) was inoculated with three drops of the homogenate, incubated at 37 °C and inspected weekly with the aid of a stereoscopic microscope for the presence of any growth. After standard bacteriological identification, all isolates were subjected to spoligotyping as described by [20]. Individual red deer were classified as 'diseased' or 'healthy'. While culture as the gold standard for TB has proven to show low sensitivity, animals negative by culture but positive for gross lesions were not included in this study.

### 2.3. Comparative cervical skin test

The 33 individuals captured by keepers were tested for TB by means of the comparative cervical skin test (CCT). Hinds were individually ear-tagged and kept in open-air enclosures before they were physically immobilised in a manual crush for measuring thickness of skin and performing TB CCT. Three areas of skin (3 cm diameter) on the right side of the neck separated by 4 cm each, were shaved prior to intradermal injection. Each animal was held in the crush for less than 10 min. On Day 1, deer were injected with 0.1 mL of two solutions of tuberculin antigens from *M. bovis* (PPD-b) and *M. avium* (PPD-a) (Cooper-Zeltia) in separate skin areas, respectively. As a positive control, animals were also intradermally injected with phytohaemagglutinin (250 μg/mL, Sigma-Aldrich, Missouri, USA; [21]). One-mL syringes fitted with a 25-G*1/2-inch needle were used. Immediately prior to injection, skinfold thickness was measured twice, to the nearest 0.1 mm, using a digital calliper (Mitutoyo), by the same person. Blood was taken on day one. The inoculation sites were measured 72 h after injection of PPD. Any skin thickening at the PPD-b site, which was at least 2 mm greater than the reaction at the PPD-a site, was recorded as positive [15]. After the second measure, deer were euthanised and a complete necropsy and micobacterial culture were performed. We collected samples during regular TB skin testing and abattoir activities carried out by game managers; therefore, we neither designed nor were responsible for the management and culling of the animals included in this study. However, the use of animals in this research was approved on 18 March 2015 by the Research Ethics Commission of Castilla-La Mancha University, Albacete, Spain. CEEA: PR-2015-03-08.

### 2.4. Quantification of parasite infection

Since concomitant parasite infections can potentially affect individual health and Hp serum levels, parasite loads from a subsample of the individuals were determined, including the parasite taxa most commonly described in red deer across the study area [22–24]. For this purpose, fresh faecal samples were collected directly from the rectum during field necropsy. Bronchopulmonary L1 larvae ($n = 75$) were extracted in less than 24 h [25] and were expressed as the number of larvae per gram of faeces (*lpg*). *Elaphostrongylus cervi* L1 and *Dictyocaulus* spp. were morphologically identified under a light microscope [24]. The faecal flotation technique ($n = 39$) using a zinc sulphate solution [26] revealed the presence of Tricostrongylidae eggs, and subsequent counts (as number of eggs per gram of faeces, *epg*) were performed with a McMaster camera. The head, neck, ears and ventral surface of animals ($n = 28$) were inspected to quantify tick burden (Ixodidae; [22]). Finally, to quantify pharyngeal bot fly larvae (*Cephenemyia auribarbis* and *Pharyngomyia picta*, Oestridae; [23]) ($n = 33$), the head, trachea and lungs were dissected.

### 2.5. Determination of serum haptoglobin concentration

The commercial kit Tridelta Phase range serum Hp (Tridelta Development Limited, Ireland) was used to determine Hp according to the manufacturer's instructions. This assay is based on the finding that the formation of the haemoglobin-haptoglobin complex preserves the peroxidase activity of haemoglobin against inactivation at a low pH. All determinations were performed in duplicate in the automated biochemistry analyser Cobas Mira Plus (ABX Diagnostic Montpellier, France). The assay was preliminarily validated in the laboratory before being applied in deer samples. For this purpose, precision, accuracy and limit of detection were calculated. Within-run coefficients of variation (CVs) were calculated after the analysis of 3 serum samples with low, medium and high Hp concentrations five times in a single run. Between-run CVs were obtained by measuring the same samples in five separate runs carried out on five different days. All samples used were frozen in aliquots and only the vials needed for each run were used. Accuracy was investigated by linearity under dilution; in brief, two deer serum samples were serially diluted (1:2; 1:4; 1:8; 1:16) with saline solution. The limit of detection was calculated as the lowest concentration of Hp that could be distinguished from a zero sample, and was taken as the mean +2 standard deviations (SD) of 12 replicates of blank sample, tested in one analytical run.

### 2.6. Statistical analysis

Before testing for the effect of TB infection on Hp serum levels, we explored the potential effects of concomitant parasite loads on Hp levels, which could mask any effect due to TB infection. For this purpose, we performed separate statistical analyses for a range of different parasite taxa. We designed Generalised linear models (GLM) including Hp serum concentration as the continuous response variable, and TB status (binomial categorical variable) and respective parasite load (continuous variable) as explanatory variables. After discarding any effect of parasite loads, we tested for the effect of TB status (as a binomial categorical explanatory variable: negative, $n = 53$, and positive, $n = 36$) on serum Hp concentration (as a response variable), with a GLM ($n = 88$). We also included management (categorical binary explanatory factor, 0 = managed for CCT and euthanised, $n = 33$; and 1 = shot by hunters, $n = 55$) and its interaction with TB status. We used a Normal error and a log link ($P < 0.05$ at Kolmogorov-Smirnov test for normality of Hp concentration). As a reference, the range for normal values of serum Hp was considered within 5 $^{th}$ and 95 $^{th}$ percentiles in TB negative animals at post-mortem. This range was used to infer the diagnostic value of Hp serum concentrations, so that individuals with levels above the upper limit were considered to have a HP serum concentration compatible with TB positive status. For evaluation of sensitivity and specificity of HP levels, as a complement to CCT, we used the post-mortem analyses (gross lesions confirmed by culture) as the gold standard to classify individuals as TB negative or positive. Statistical significance was assumed wherever $P \leq 0.05$. We employed the Statistica 6.0 (StatSoft Inc, 2001) statistical package.

## 3. Results

### 3.1. Diagnosis of tuberculosis infection, CCT results and quantification of parasite infection

Post-mortem examination showed that 36 out of 88 animals were TB positive (40.1 ± 5.22% of the study animals). Parasite infection status for *E. cervi* L1, *Dictyocaulus* spp. L1, Trichostrongylidae (epg), Oestridae larvae and Ixodidae ticks are shown in Table 1. Eight out of 33 CCT tested animals were positive at the post-mortem analyses, but CCT suggested that 6 of them were TB negative (the differences of skin thickening between PPD-b and PPD-a sites were smaller than 2 mm), and therefore were classified as false negatives; skin thickness measures and Hp levels for these animals are shown in Table 2. The sensitivity and specificity of CCT in relation to post-mortem analysis as a gold standard, and the proportion of false positives and false negatives are shown in Table 3. CCT showed low sensitivity and high specificity. Concerning the individuals that were negative upon post-mortem examination, 2 out of 25 were positive for the CCT (false positives).

### 3.2. Analytical validation of the Hp assay

Within-run and between-run CVs are presented in Table 4. Mean within-run and between-run CVs were 3.8% and 6.6%, respectively. Dilution studies resulted in linear regression equations with a correlation coefficient of 0.99 showing that the method measures the protein in a linear manner (Fig. 1). The detection limit of the assay was 0.02 *g*/L.

### 3.3. Hp in relation to handling, parasite and tuberculosis infections

No statistical relationships between Hp serum levels or any parasite abundance were evidenced ($P > 0.05$ in all cases) after controlling for TB status (Table 1). Concerning the model testing the effect of TB status on Hp serum levels, Hp was significantly higher in individuals positive for TB (Tables 5 and 6). Hp mean and median values, and the ranges defined by the lower and upper quartiles (within parenthesis, respectively), were 0.52 and 0.23 (0.17–0.42) *g*/L for healthy individuals and 1.69 and 0.39 (0.24–2.49) *g*/L for infected individuals (Fig. 2a. shows the boxplots). As a reference range, the 5th and 95th percentiles for serum Hp in animals negative for TB upon post-mortem examination were 0.14 and 2.40 *g*/L, respectively. In addition, there was a significant statistical association between handling and Hp concentrations (Tables 5 and 6, Fig. 2b.), showing that captured and handled animals had higher serum Hp levels than those that were hunted (Hp levels ± SD were 1.21 ± 1.64 *g*/L, $n = 33$ for managed deer; and 0.87 ± 2.19 *g*/L, $n = 55$ for hunted deer, Fig. 2b).

### 3.4. Hp in relation to CCT

Data from the comparison of the Hp concentrations and CCT results for the detection of TB animals are presented in Tables 2 and 3. Four of the 6 animals that were CCT negative but had TB exhibited high levels of serum Hp, which were higher than the 95th percentile for healthy individuals (2.40 *g*/L). In particular, one out of this four false negatives with high levels of Hp was anergic, as a positive control for skinfold thickness increase was 0 mm (it ranged from 10 mm to 41 mm in the remaining false negatives). The two individuals negative upon post-mortem examination that were positive based on the CCT presented Hp levels of 0.15 and 3.22 *g*/L, the latter clearly above the 95th percentile for healthy individuals (2.40 *g*/L). Only one out of the 23 (4.35%) animals negative for both post-mortem and CCT analyses showed Hp serum levels (6.80 *g*/L) clearly above the 95th percentile.

## 4. Discussion

To the best of our knowledge, this is the first report that describes Hp serum values in wild animals considering infection status. A positive association between circulating serum Hp and TB infection in red deer was observed. This finding suggests that an acute phase response (in terms of Hp levels) develops in infected animals. Our results are in line with previous research in humans, which suggested that changes in APP (including an increase in Hp) in *M. tuberculosis* infected patients are innate unspecific responses due to mycobacteria-induced tissue damage [27].

Hp may play a functional role against disease. Evidence of this includes the fact that Hp polymorphisms in humans play a role in the clinical course of tuberculosis [28,29]. The mechanisms may relate to preventing pathogens from utilising iron of iron-containing proteins

**Table 1**
Parasite infection figures (prevalence ± SE, abundance ± SD) for *Elaphostrongylus cervi* (*lpg*); *Dictyocaulus* spp. (*lpg*); Tricostrongylidae (*epg*); Oestridae larvae counts; and Ixodidae tick counts. This table also shows Hp serum level (*g*/L) ± SD as a function of the parasite infection status (negative *vs* infected). No statistical relationships between Hp serum levels and abundance of any parasites were evidenced (*P*-value always > 0.05) after controlling for TB status.

| Parasite (*n*) | Prevalence (%) | Abundance | Hp in animals with the parasite | Hp in animals without the parasite |
|---|---|---|---|---|
| *E. cervi* (*lpg*, $n = 75$) | 66.67 ± 5.48 | 23.70 ± 44.7 | 0.68 ± 1.17 | 1.51 ± 3.18 |
| *Dictyocaulus* spp. (*lpg*, $n = 75$) | 12.00 ± 3.78 | 3.08 ± 10.85 | 1.40 ± 2.08 | 0.89 ± 2.21 |
| Tricostrongylidae (*epg*, $n = 39$) | 20.51 ± 6.55 | 6.37 ± 11.2 | 0.75 ± 1.53 | 0.98 ± 0.99 |
| Oestridae larvae ($n = 33$) | 15.15 ± 6.34 | 4.53 ± 10.2 | 0.41 ± 1.07 | 0.66 ± 0.14 |
| Ixodidae ticks ($n = 28$) | 75.00 ± 8.33 | 4.82 ± 5.34 | 0.66 ± 0.68 | 0.54 ± 1.05 |

**Table 2**
Hp levels (g/L) for animals positive for TB ($n$ = 8) upon post-mortem examination in which a comparative cervical skin test was performed ($n$ = 33). (ΔPPD-b)-(ΔPPD-a) refers to the difference between skin thickening increases (mm) at the PPD-b and the PPD-a sites, which should be at least greater than 2 mm to be recorded as positive. The Hp levels reference range was calculated as the percentiles 5% and 95% (0.14–2.40, respectively) of animals negative for TB upon post-mortem examination. Therefore, Hp levels and CCT agreed in two individuals (ind. 3 and 8); Hp levels would improve the sensitivity of CCT in 4 animals (ind. 1, 5, 6 and 7), and finally, Hp levels did not reveal two CCT positive cases (ind. 2 and 4).

| | Ind. 1 | Ind.2 | Ind. 3 | Ind. 4 | Ind. 5 | Ind. 6 | Ind. 7 | Ind. 8 |
|---|---|---|---|---|---|---|---|---|
| (ΔPPD-b)-(ΔPPD-a) (mm) | 1.3 | 2.7[a] | 1.3 | 2.7[a] | 0.9 | 1.7 | 0.5 | 0.6 |
| Haptoglobin (g/L) | 2.96[b] | 2.06 | 0.27 | 0.41 | 3.64[b] | 2.64[b] | 5.12[b] | 1.60 |

[a] Positive comparative cervical skin tests.
[b] Hp levels out of 5th and 95th percentiles.

that are released as a result of tissue damage. This would explain why an increase in Hp scales with the degree of lesion size, as more iron-binding capacity is needed. A significant decrease in the Hp concentrations (together with other positive APPs) after treatment of tuberculosis-infected human patients has also been reported [30].

APP values (including Hp) have been studied in farmed red deer in relation to general health status, as well as its reaction against an intradermal injection of bovine purified derivative (PPD) in red deer with a history of TB infection [15,16]. In these studies, it was suggested that APP levels may therefore indicate the presence of subclinical tissue damage, inflammation or infection, and significant increases in plasma Hp in response to a normal cervical skin test in TB-infected red deer were found. Differences in determination methods between [15] (Tetraguaicol method) and our study may be consequential, and should be considered when comparisons are made.

Handled deer showed higher values of serum Hp than hunted ones, which suggests that when establishing Hp reference values, the effects of animal handling should be taken into account. As suggested by the data obtained in domestic animals, such as dogs and cattle [31,32], we could postulate that invisible trauma and/or high stress with an increase in cortisol, which would increase Hp during management, could explain this finding; however, additional studies are needed to elucidate this aspect. In relation to other potential factors that could influence Hp levels in our study, we found no statistical relationship between parasite loads and Hp levels. This finding is not surprising since parasite loads were generally low and no evidence of parasite-induced lesions was found at necropsy. Additional studies including higher sample sizes and/or more controlled experimental conditions, would be required to assess if Hp levels can be influenced by more severe parasite burdens in red deer.

Although APP responses in animals are highly unspecific, they can be highly sensitive to differentiating between healthy and ill animals. TB is the most prevalent infectious disease in red deer in our study area (unpublished data), and is also the most prevalent infectious disease in apparently healthy individuals randomly sampled [17]. Therefore, recommendations about control prior to the movement of wild ungulates and quarantine measures in the area to which they are translocated are still needed. It is necessary to develop and improve diagnostic tests [33–35] since there is lack of sensitivity in tests used for in vivo

**Table 4**
Within-run and between-run variation in serum samples from deer with different Hp concentrations (g/L). SD: standard deviation; CV: coefficient of variation.

| | WITHIN-RUN | | | BETWEEN-RUN | | |
|---|---|---|---|---|---|---|
| | Mean | SD | CV | Mean | SD | CV |
| High | 9.96 | 0.20 | 2.01 | 9.78 | 0.5 | 5.11 |
| Medium | 2.11 | 0.09 | 4.47 | 2.32 | 0.16 | 6.89 |
| Low | 0.18 | 0.008 | 4.86 | 0.19 | 0.015 | 7.89 |

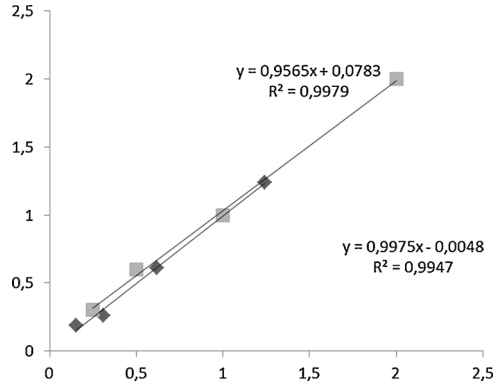


**Fig. 1.** Linearity under dilution of two serum samples.

**Table 5**
Sampling distribution and mean serum haptoglobin levels (g/L) in relation to management (captured or hunted) and TB infection status.

| Management | TB status | $n$ | Hp ± SD(g/L) |
|---|---|---|---|
| Hunted ($n$ = 55) | Healthy | 28 | 0.31 ± 0.18 |
| | Tuberculous | 27 | 1.45 ± 3.05 |
| Captured ($n$ = 33) | Healthy | 24 | 0.78 ± 1.48 |
| | Tuberculous | 9 | 2.38 ± 1.55 |

**Table 3**
Comparative Cervical Test (CCT) in relation to TB status at post-mortem examination (gold standard) in animals subjected to both tests ($n$ = 33). We also show the relevance of Hp serum concentrations in relation to the sensitivity and specificity of CCT. Finally, we show the sensitivity and specificity of serum Hp. The reference range was calculated as the percentiles 5% and 95% (0.14–2.40 respectively) of animals negative for TB upon post-mortem examination.

| TB* | CCT | Sensitivity Specificity CCT | Hp considerations | Sensitivity Specificity Hp |
|---|---|---|---|---|
| +$n$ = 8 | +$n$ = 2 | True positives Sensitivity: 2/8 | Both CCT + showed serum Hp levels < than reference range[a] | Sensitivity 5/8 |
| | –$n$ = 6 | False negatives | 4 out of 6 CCT- showed serum Hp levels > reference range, one being anergic to the positive control at CCT* | False negatives 3/8 |
| –$n$ = 25 | +$n$ = 2 | False positives | 1 out 2 CCT + showed serum Hp levels > than upper reference value | False positives 2/25 |
| | –$n$ = 23 | True negatives Specificity: 23/25 | 1 out of 23 CCT- showed serum Hp levels > than the upper reference range | Specificity 23/25 |

[a] For animals positive for TB upon post-mortem examination, the Table 5 shows the skin thickness measures and Hp levels (g/L).

**Table 6**
Models for the association between haptoglobin serum levels (dependent variable), tuberculosis status of red deer management (captured versus hunted) and their interaction ($n = 88$). Parameter estimates ($\beta$) for the level of fixed factors were calculated using a reference value of 0 for the 'managed' level in the variable 'Management', and for the 'positive' level in the variable 'Tuberculosis'.

| | df | Wald $Chi^2$ | Parameter estimates ($\beta \pm$ S.E.) | $P$ |
|---|---|---|---|---|
| Tuberculosis | 1 | 16.37 | Negative = −0.59 ± 0.15 | < 0.01 |
| Management | 1 | 10.69 | Managed = −0.47 ± 0.14 | 0.001 |
| TB*Management | 1 | 3.86 | Managed*Negative TB = 0.35 ± 0.18 | 0.05 |

diagnosis of TB infection in the field, such as CCT [9,36]. Information provided by Hp levels could complement CCT tests in red deer by increasing their sensitivity. For example, in our study, by using the criteria that any animal with an increase in Hp and/or a positive CCT is TB-positive, sensitivity would increase from 25% (2/8) to 75% (6/8). However, this criterion would reduce specificity because two healthy deer (according to the gold standard) out of 25 presented serum Hp levels over the aforementioned upper range. This reduced specificity may be due to the fact that Hp can also increase with other infectious diseases, after an inflammatory stimulus or stress.

## 5. Conclusions

We suggest that (i) a measure of Hp serum levels may be a good indicator of general health status in red deer and (ii) abnormal increased Hp values with a normal CCT test could indicate that the animal is at risk for a subclinical TB infection, not detected by CCT. Therefore, the combined use of CCT and Hp should increase sensitivity to detection of TB-infected animals. This approach reduces specificity, but would largely reduce sanitary risks during translocations (or other practices of wildlife management requiring in vivo diagnostics).

Hp determination was shown to have the potential as a complementary method to improve the sensitivity of conventional specific diagnostic tests in wildlife, which confer a practical value to its use and presents a new field of research in APP applications. However, these results are only preliminary and should be considered with caution. Further efforts should be made to identify a larger number of unhealthy TB-free animals to improve our interpretation of HP levels as a complement to TB diagnostic tests. Future research focusing on more complete studies including a higher number of animals, the use of APP profiles including various APPs and the calculation of critical difference values for APPs in deer serum samples, will significantly contribute to a better understanding of the APP reaction and a better interpretation of APP results in red deer and wildlife ruminants in general.

## Conflicts of interest

None.

## Acknowledgments

This work was supported by the Spanish Ministry of Economy and Competitiveness [AGL2013-48523-C3-1-R]. We thank the Spanish Organism of National Parks. We also wish to thank the students and colleagues at IREC for their help with the laboratory and field work. Jordi Martínez-Guijosa holds a FPI pre-doctoral scholarship [BES-2015-072206], funded by the Spanish Ministry of Economy and Competitiveness.

Preliminary results were presented as a poster and oral presentation at the 6th Reunião sobre Ungulados Silvestres Ibéricos, São Pedro do Sul, Portugal, 4th–5th September 2015.

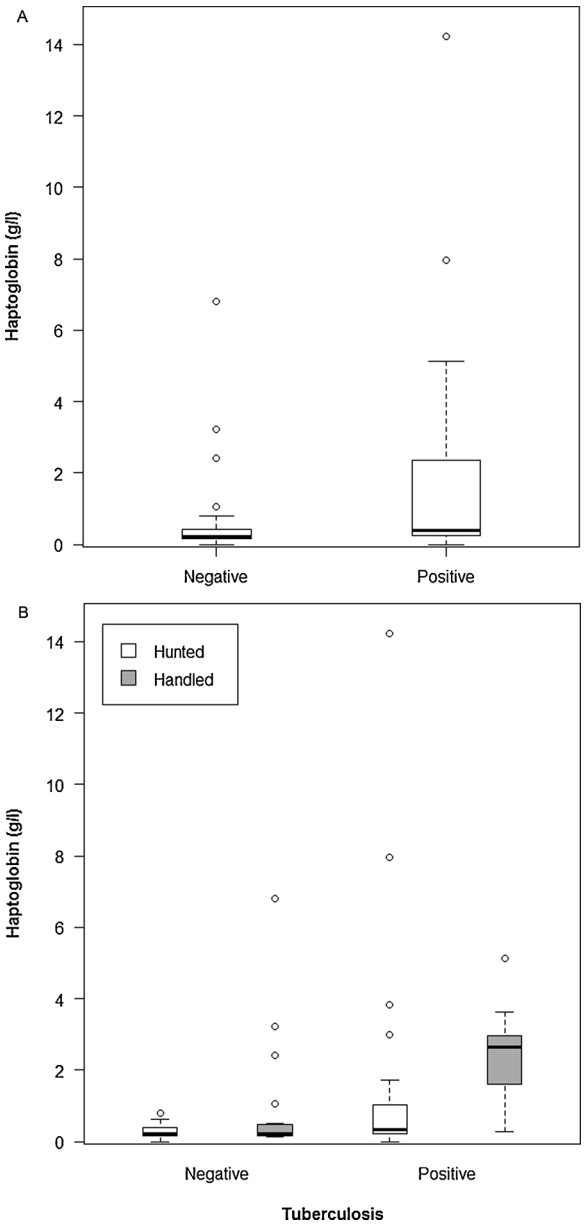


**Fig. 2.** (a) Boxplot for serum haptoglobin (Hp) concentration (g/L) in red deer in relation to TB infection status (the box draws from the 25th to the 75th percentile). The box is split with a line at the median, and the whiskers shown above and below the boxes represent the largest and smallest observed scores that are less than 1.5 box lengths from the end of the box. The open circles are scores (outliers) that are more than 1.5 box lengths. The highest outliers for TB positive deer are shown outside of the axis scale and values are indicated within brackets. (b) Serum Hp concentration (g/L) in red deer ± SD in relation to handling (hunted deer versus captured handled deer) and TB infection status (0 = non-tuberculous and 1 = tuberculous).

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