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Evaluation of Hyaluronic Acid in Cattle: Physiological Variations Related to Age, Periparturition and in Clinical Cases of Paratuberculosis

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Abstract

Hyaluronic acid (HA) serum levels have been related to various pathological conditions in both humans and animals. The involvement of HA in the pathogenesis of mycobacterial infections has been suggested. The aim was to evaluate serum levels and ilea tissue presence of HA in healthy bovines and in clinical paratuberculosis (PTB) cases. HA serum concentrations in bovines were found to be in the range of 130 to 617 ng/mL with a median value of 418 ng/mL. Significantly increased HA levels were detected in 1 month-old calves. Increased levels of HA were found at peripartum as compared to healthy control animals, with pre- parturition values significantly higher than post- parturition (p=0.02). Cows with clinical PTB showed higher serum levels and diminished ileal detection of HA than healthy animals. In serum, physiological variations in HA serum levels related to age and parturition were found in bovines. In addition, clinical PTB also affected the presence of HA in serum and ileum. These results might contribute to elucidate the clinical significance of HA evaluation in cattle and its involvement in PTB infection.

Keywords: Bovine; Hyaluronic acid; Age; Parturition; Paratuberculosis

Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a polysaccharide found in all tissues and body fluids of vertebrates [1]. Circulating HA is synthesized at the plasma membrane by a group of highly specialized proteins, HA synthases (HASs). Three well-conserved HAS isozyme present in mammalian species: HAS1, HAS2 and HAS3 have been described [2]. The best characterized transmembrane hyaluronan receptor is CD44. This receptor is a widely distributed surface glycoprotein expressed on most cell types and it is considered to be a highly conserved molecule among mammals [3]. The removal of HA from the blood mainly takes place by hepatic hyaluronidases, where receptor-facilitated uptake occurs in the sinusoidal endothelial cells [4]. The role of HA in physiological and pathological states such as embryo development; migration, adhesion, proliferation and differentiation of cells, immune surveillance, immune regulation, inflammation, wound healing, angiogenesis, multi-drug resistance, and tumorigenesis is well documented [5-11]. Altered HA concentrations in blood have been reported to be related to various pathological conditions in humans [12-14]. In veterinary medicine, HA levels have been evaluated as a biomarker in different diseases, such as osteoarthritis [15,16], acute abdominal pain [17], cutaneous mucinosis [18], and liver pathology [19,20] in dogs and horses. To our knowledge, there is only one report indicating the physiological levels of serum HA in cattle, where a radio assay was used to assess HA levels [21]. To date, no reports were found in the literature regarding variations of HA levels in paratuberculosis (PTB), a chronic enteric disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). Nevertheless, the involvement of HA in mycobaterial pathogenesis has been first suggested as early as in 1977, when Lee and co-workers described an improvement in mycobacterial growth in media supplemented with hyaluronate [22]. More recently, it has been described that pathogenic mycobacteria use HA to invade [23] and to grow in the respiratory tract *in vivo* [24,25]. Moreover, hyaluronidase activity has been detected in mycobacterial cultures while the *Rv0394c* gene encoding a hyaluronidase in *Mycobacterium tuberculosis* has been characterized [26]. Experimental evidences for a potential role of CD44 receptor in the immune response to *M. tuberculosis* have been described by different authors [27-29].

The aim of this work was to evaluate HA levels in healthy bovines and to compare them with PTB clinically infected cattle. We found HA serum levels variations that were related to age, periparturition and infection in cattle. HA tissue detection was altered in PTB cases. These results might contribute to elucidate the clinical significance of HA evaluation in cattle and its involvement in PTB infection.

Materials and Methods

Ethics statement

All animals employed in this work were handled according to the protocols approved by the Institutional Committee for the care and use of experimental animals of the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires.

Animals

A total of 31 female Holstein-Frisian bovines from commercial farms were included in this study and categorized as:

- Healthy cows (n=20), classified by clinical and pathological examinations, which had been raised in tuberculosis-free accredited and *Map*-free dairy herds of the Pampas region of Argentina. This group included: 10 cows, with ages ranging from 1 to 6 years; 3 calves, which were serially sampled at 1, 4 and 6 month of age (healthy controls) and 7 periparturient cows, that were examined 20 days around parturition.

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- Clinical PTB-infected cows (n=11), which had been raised in herds with history of PTB, with ages ranging from 2 to 6 years. Briefly, animals were examined for clinical signs of disease (cachexia, chronic diarrhea) and for the presence of *Map* in milk and/or feces. Diagnosis was confirmed by mycobacterial isolation in Herrold's egg yolk medium with mycobactin J and amplification of the IS*900* fragment by PCR [30]. All diagnostic techniques were conducted following recommendations of the OIE [31].

Samples

Sera: Blood samples were obtained from coccygeal venipuncture of adult bovines. The three calves studied were bled from the jugular vein at 1, 4 and 6 month of age. The seven periparturient cows were bled twice: around 7 days pre- and between 4 and 13 days post-parturition. Serum samples were obtained by centrifugation of whole blood and stored at -20°C before HA measurement.

Intestinal specimens: Complete necropsies were performed from 4 cows (2 from the healthy and 2 from the clinical PTB group), and tissues were visually examined with special attention to the gut. Macroscopic lesions were documented and samples from the distal ileum were collected for histology and histochemistry. Tissues were fixed in 10% neutral buffered formalin and routinely processed for hematoxylin/ eosin (HE) and Ziehl-Neelsen (ZN) stainings. Microscopic lesions consistent with *Map* infection were classified following the guidelines previously proposed by González et al. for PTB lesions in bovines [32].

Assessment of HA levels in serum by ELISA

As the structure of HA is conserved among different animal species, HA levels in sera were measured by a competitive Enzyme-Linked Immunosorbent Assay (ELISA) as described previously [10]. Briefly, 96 well microtiter plates were coated with 100 μg/mL HMW-HA (High molecular weight hyaluronic acid; CPN spol. s.r.o Czech Republic, Farmatrade Argentina) at 4°C. Samples and standard HMW-HA were incubated with 0.5 μg/mL biotinylated HA binding protein (bHABP) (#385911, Calbiochem) at 37°C. The plate was blocked and incubated with samples at 37°C for 4 h. The bHABP bound to the wells was determined using an avidin-biotin detection system (# A2004, Vector). Sample concentrations were calculated from a standard curve.

Evaluation of ileal HA by Histochemistry

Hyaluronan staining was performed as described previously [6]. After deparaffination, the endogenous peroxidase activity was quenched by incubation slides with 3% H_2O_2 in methanol for 15 min at room temperature (RT). To block non-specific binding sites, sections were incubated with 10% fetal bovine serum in PBS for 30 min at RT and an avidin and biotin blocking solution (Vector, Peterborough, UK). Then byotinilated hyaluronan binding protein (bHABP) (Calbiochem, Darmstadt, Germany) was added and incubated overnight. As amplification and revealing system, an avidin-peroxidase complex (Vector, Peterborough, UK) was used. The reaction product was visualized by the addition of a diaminobenzidine solution (DAB) (Vector, Peterborough, UK) followed by counterstaining with Mayer's hematoxylin. To assess non-specific binding of bHABP tissue sections were either stained with bHABP that had been pretreated with 50 U/ml hyaluronidase from bovine testes (Sigma, USA) in PBS at 4°C overnight or incubated without the addition of bHABP.

Statistical analysis

Individual results are expressed as mean ± standard deviation of two independent measures, run in duplicate. Group results are expressed as median HA levels, with the minimum and the maximum value of the range between brackets. Data were processed with the STATISTIX 8.0 software (Analytical Software, Tallahassee, FL, USA). Serum HA concentrations were not normally distributed and were therefore analyzed using the non-parametric Kruskal-Wallis test. For analysis of parturition effect on HA levels, pre- and post- parturition values were compared using the Wilcoxon Signed Rank Test. A P value<0.05 was considered statistically significant.

Results

As a first step, we evaluated the concentration of HA in the sera of 10 healthy cows, with ages ranging from 1 to 6 years. HA concentrations ranged from 130 to 617 ng/mL, with a median value of 325 ng/mL (Figure 1A). As variations in serum HA levels with age have been reported in humans [33], we decided to serially assess HA levels in sera from healthy calves at 1, 4 and 6 months of age. The levels detected at 1 month resulted significantly higher than those found at 4 and 6 months (Figure 1B). HA levels corresponding to 6 month calves were included in those data obtained for adults for further analysis. Then, the median value finally considered for the healthy median range was 418 ng/mL.
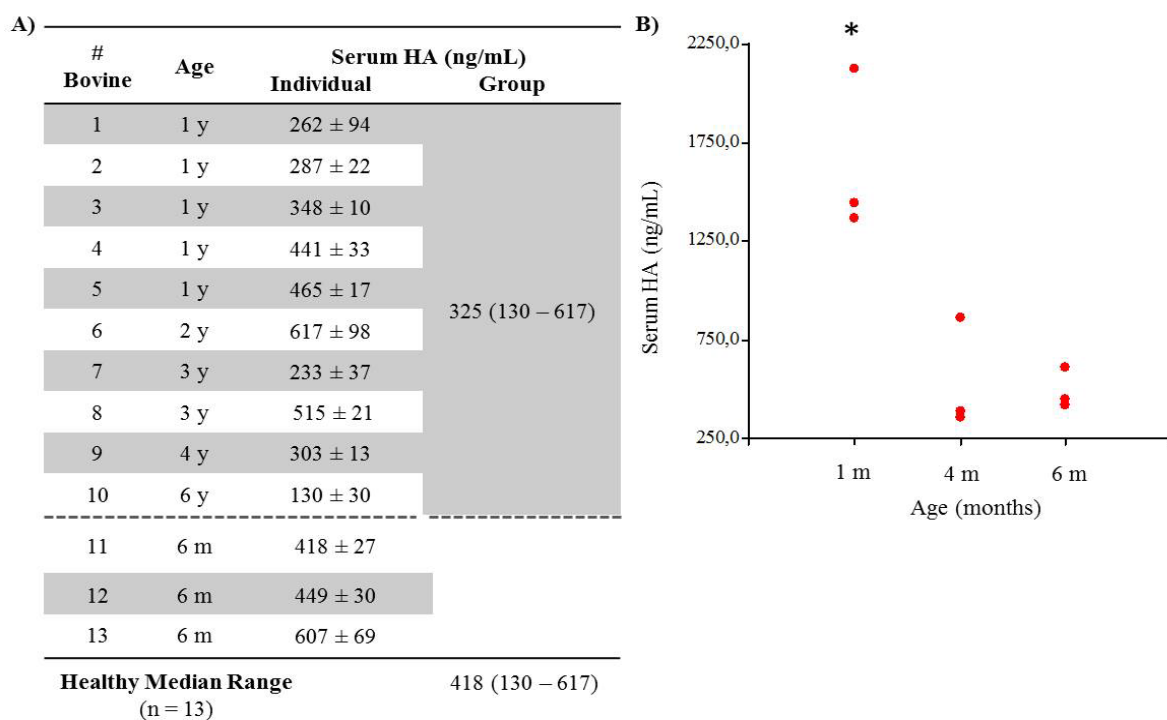
When HA levels at parturition were analyzed, we found that all periparturient cows showed increased HA levels regardless of the sampling time (pre- or post-parturition). These values were even higher than the maximum value observed for healthy controls (Table 1). In all cases, HA pre- parturition values were higher than those obtained at post- parturition (p=0.02).

Animals with clinical PTB had a statistically significant increase in HA serum levels, as compared with healthy cows (range: 443-1471 ng/mL, median value: 656 ng/mL, p=0.004) (Figure 2). Two animals from each of these groups were euthanized and comparative histopathological and histochemical analyses were conducted on distal ileal tissue. Macroscopic histopathological lesions included mucosal thickening, predominantly in the distal small intestine and enlargement of mesenteric lymph nodes. Microscopic examination of HE stained ilea revealed lesions consistent with diffuse multibacillary paratuberculosis, with loss of tissue architecture due to the presence of an inflammatory infiltrate, mainly made up of macrophages. These histopathological changes were found in both mucosa and submucosa (Figure 3B). ZN staining of ileum sections revealed the presence of acid-fast bacilli (AFB) (Figure 3D and D´). Neither histopathological findings nor evidences of AFB were detected in tissue sections from healthy cows (Figure 3A and 3C).

To determine if the presence of HA was modified at the site of infection in PTB clinical cases, we conducted a HA histochemical evaluation. Differences in the intensity and extent of labeling were found. In ileal tissue belonging to healthy cows, HA was found to be broadly distributed in both, the mucosa and submucosa (Figure 3E). Contrarily, HA labeling was found to be diminished in specimens from clinical PTB cows, mainly in submucosa cells (Figure 3F).

Discussion

Circulating HA levels have been well documented in healthy humans, with concentrations ranging from 10 to 100 ng/mL. In animal species such as rat, rabbit, dog, pig, goat, sheep, cow and horse, HA concentrations have been found to be similar or higher than those found in humans [21]. The data from healthy cows reported in this study contribute to the limited knowledge of normal reference ranges of serum HA in bovines. Our results were in agreement with the only previous HA reference values available in the literature, assessed by

A)

# Bovine	Age	Serum HA (ng/mL) Individual	Serum HA (ng/mL) Group
1	1 y	262 ± 94	
2	1 y	287 ± 22	
3	1 y	348 ± 10	
4	1 y	441 ± 33	
5	1 y	465 ± 17	325 (130 − 617)
6	2 y	617 ± 98	
7	3 y	233 ± 37	
8	3 y	515 ± 21	
9	4 y	303 ± 13	
10	6 y	130 ± 30	
11	6 m	418 ± 27	
12	6 m	449 ± 30	
13	6 m	607 ± 69	
Healthy Median Range (n = 13)			418 (130 − 617)

Figure 1: The smart-surface constitution of superficial phospholipid bilayer of articular cartilage in: **(a)** wet and **(b)** air-dry condition. A change in surface energy leads to conformational changes in the surface phospholipids from bilayer (hydrophilic) to monolayer (hydrophobic) ones [6-8].

# Bovine	Serum HA (ng/mL)	
	Pre- parturition (-10 to -1 dpp)	Post- parturition (+4 to +13 dpp)
14	1241 ± 231	843 ± 16,1
15	656 ± 11	539 ± 33
16	1188 ± 127	750 ± 81
17	899 ± 56	534 ± 63
18	706 ± 66	598 ± 29
19	1262 ± 3	908 ± 30
20	1134 ± 20	857 ± 10
Median Range	1134.4 (656.1-1262.3) **A**	749.3 (533.6-908.7) **B**

Individual results are expressed as mean ± standard deviation of two independent measures. Group results are expressed as median HA level and range between brackets. A and B denotes statistical significance (p=0.02, Wilcoxon Signed Rank Test).

Table 1: HA levels in periparturient cows.

radio assay [21]. Similarly to other works carried out with horses [34] and dogs [35], the HA serum range described in this work for bovines (130-617 ng/mL) seems to be broader than those found in healthy humans.

We studied 13 healthy bovines from 1 month to 6 years old. Younger calves (1 month-old) had HA serum levels that were about 3 to 5 times higher than those corresponding to older animals. The latter phenomenon is also observed in humans during the first week after birth, when HA serum levels are 6 to 8 times higher than those of adults [33,36].
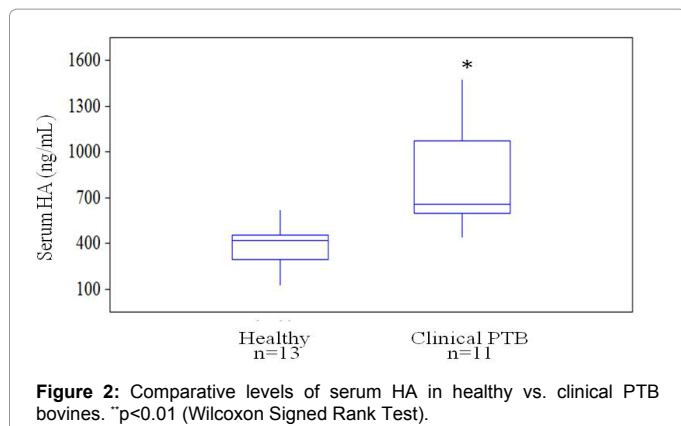
Regarding variations in HA levels in the periparturition period, the values obtained were higher than the upper limit of the range described for healthy individuals. This phenomenon has also been described for Guinea pigs [37] and women near term [38]. It has been suggested that

this increase might be associated with the mobilization of HA from the extracellular matrix of the uterine cervix into the circulation as part of cervical ripening process during parturition.

Elevated HA serum levels have been reported in several pathological conditions characterized by systemic inflammation and activation of the immune system [11]. Moreover, it has been demonstrated that HA mediates defensive mechanisms in the respiratory [9,39] and intestinal [40] mucosal surfaces. Even more, a role for HA in the pathogenesis of tuberculosis has been recently proposed by different authors [24,25,29]. In this context, we found variations of HA in sera and ilea of PTB clinical cows as compared to healthy animals. Since hyaluronidase activity has been reported in *M. tuberculosis* [26], it is possible to consider that *Map* might also present a hyaluronidase that is involved in the degradation of tissue HA. This cleavage might be considered as a first step for HA usage as a carbon source. Moreover, fragmented HA has potent proinflammatory [5] and immune protective [41] functions. Furthermore, the presence of hyaluronidases has been demonstrated in human monocytes [42]. Taking these data into account it can be hypothesized that infiltrating macrophages found in ileal samples of PTB clinical cows could have similar hyaluronidase activity, generating HA fragments responsible for the diminished HA signal detected. Other hypothesis to explain the weaker HA signal in the infected tissues could be related to the possible production of reactive oxygen species by infiltrating mononuclear cells. These inflammatory mediators have been found to be involved with HA degradation in some disease models such as arthritis [43].

The decreased HA tissue signal described herein for PTB clinical cases differs from the findings reported for Crohn's disease [44] and in *M. bovis*-induced acute granulomatous lesions in monkeys [24]. The fact that differences in HA intensity and extent of labeling were found when comparing ileal tissues of PTB-infected and healthy cows

Figure 2: Comparative levels of serum HA in healthy vs. clinical PTB bovines. ¨p<0.01 (Wilcoxon Signed Rank Test).

suggests that variations in HA serum levels in such group might be related to this pathology.
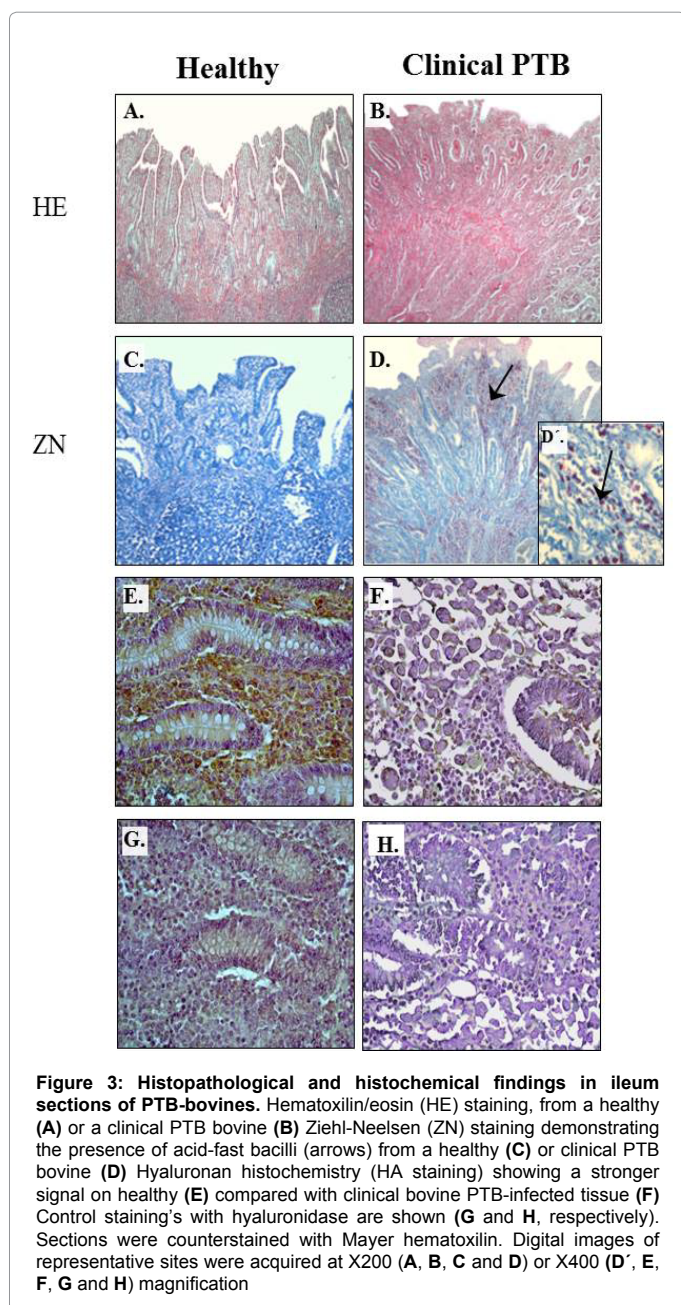
In summary, we conclude that the range of HA serum levels in bovines is broader than that previously reported for healthy humans, with physiological variations related to age and parturition. In addition, clinical PTB also affected the presence of HA in serum and ileum. The biological significance of HA evaluation in PTB infection in cattle remains to be elucidated.

Acknowledgements

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Figure 3: Histopathological and histochemical findings in ileum sections of PTB-bovines. Hematoxilin/eosin (HE) staining, from a healthy **(A)** or a clinical PTB bovine **(B)** Ziehl-Neelsen (ZN) staining demonstrating the presence of acid-fast bacilli (arrows) from a healthy **(C)** or clinical PTB bovine **(D)** Hyaluronan histochemistry (HA staining) showing a stronger signal on healthy **(E)** compared with clinical bovine PTB-infected tissue **(F)** Control staining's with hyaluronidase are shown **(G** and **H**, respectively). Sections were counterstained with Mayer hematoxilin. Digital images of representative sites were acquired at X200 (**A**, **B**, **C** and **D**) or X400 (**D´**, **E**, **F**, **G** and **H**) magnification

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