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Research Paper

Relationship between Paratuberculosis and the microelements Copper, Zinc, Iron, Selenium and Molybdenum in Beef Cattle

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Abstract

To study the deficiency of minerals and its relationship with Paratuberculosis, blood, serum, and fecal samples were obtained from 75 adult bovines without clinical symptoms of the disease and from two bovines with clinical symptoms of the disease, from two beef herds with a previous history of Paratuberculosis in the Province of Buenos Aires, Argentina. Serum samples were processed by ELISA and feces were cultured in Herrolds medium. Copper, zinc and iron in serum were quantified by spectrophotometry and selenium was measured by the activity of glutathione peroxidase. We also determined copper, zinc, iron and molybdenum concentrations in pastures and the concentration of sulfate in water. *Mycobacterium avium* subsp *paratuberculosis (Map)* was isolated from 17.3% of fecal samples of asymptomatic animals and from the fecal samples from the two animals with clinical symptoms. All the *Map*-positive animals were also ELISA-positive or suspect, and among them, 84.6% presented low or marginal values of selenium and 69.2% presented low or marginal values of copper. The two animals with clinical symptoms, and isolation of *Map* from feces and organs were selenium-deficient and had the lowest activity of glutathione peroxidase of all the animals from both herds. All the animals negative to *Map* in feces and negative to ELISA had normal values of Se, while 13.8% of animals with positive ELISA or suspect and culture negative presented low levels of Se. Half of the animals that were negative both for ELISA and culture in feces were deficient in copper but none of them presented low values of selenium. The content of molybdenum and iron in pasture was high, 2.5 ppm and 1.13 ppm in one herd and 2.5 ppm and 2.02 ppm in the other, respectively, whereas the copper:molybdenum ratio was 1.5 and 5.2, respectively. These results do not confirm an interaction between imbalances of the micronutrients and clinical Paratuberculosis, but show evidence of the relationship between selenium deficiencies in animals with *Map* infection and ELISA positive results.

Key words: Paratuberculosis, micronutrients, selenium, molybdenum, copper.

Introduction

Paratuberculosis is a chronic infectious disease caused by *Mycobacterium avium* subsp *paratuberculosis* (*Map*), which affects many domestic species such as cattle, sheep, and goats, and wild species such as foxes, deer and hares. Paratuberculosis is a common disease very frequent in dairy and beef cattle in Argentina (Paolicchi *et al.*, 2003) and in all countries with a significant dairy industry, especially in areas with a moderate and humid climate (Barkema *et al.*, 2010; Manning and Collins, 2001). Infected cows may have clinical signs such as persistent diarrhea which leads to the animal's weight loss. The infected ani-

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mals containing millions of *Map* in their stools contaminate the pasture, and asymptomatic animals may also shed *Map* in colostrum and milk (Streeter *et al.*, 1995). In Argentina, estimates from some regions of the country show seroprevalence percentages that vary between 7% and 19% in beef herds (Paolicchi *et al.*, 2003). Particularly in dairy cattle, losses of milk production and poor body condition followed by death or culling are predominant (Hasanova and Pavlik, 2006). The economic losses due to Paratuberculosis in Argentina are estimated to be 22.0 million dollars for breeding cattle and and 6.3 million dollars for dairy cattle in the breeding livestock area of Buenos Aires province (Paolicchi *et al.*, 2003).

Map is characterized by slow growth in suitable culture media and dependence on mycobactin. The most important difference between *Map* and other mycobacteria is the repetitive and specific presence of the IS*900* insertion sequence (Green *et al.*, 1990). The conclusive diagnosis of Paratuberculosis requires the isolation of *Map* from feces, milk, semen or affected tissues from animals with clinical symptoms (Whitlock, 1998). Although ELISA tests detect circulating antibodies in serum from infected animals, the possibility to identify infected animals increases when they have clinical symptoms of the disease. In addition, the sensitivity of this test increases when the animals excrete high quantities of *Map* through feces. This coincides with periods that compromise the immune response of the animal like management in intensive exploitations, the stress of suckling, transport, nutritional alterations, and mineral deficiencies (Manning and Collins, 2001).

Causes of mineral deficiency are poor concentration of micronutrients in the diet (primary deficiency) or inability of the animal to absorb minerals from the diet, even when the concentration of micronutrients in the diet is normal (secondary deficiency) (Wikse *et al.*, 1992). Deficiency of some minerals can affect the immunological system, diminishing the animal's capacity to overcome infections. Complex inter-relationships exist between certain micronutrients, immune function and disease resistance in cattle. Several micronutrients have been shown to influence immune responses. Deficiencies of copper (Cu), selenium (Se), vitamin E and cobalt (Co) in cattle reduce the ability of isolated neutrophils to kill yeast and/or bacteria. Cu deficiency reduces antibody production, but cell-mediated immunity is generally not altered (Arthington, 2006). However, Cu deficiency appears to reduce production of interferon and tumor necrosis factor by mononuclear cells. Numerous studies have linked low vitamin E and/or Se status to increased susceptibility of dairy cows to intramammary infections (Arthington, 2006; Spears, 2000).

Cu is an important mineral in cattle nutrition and its deficiency causes a decrease in the resistance to diseases, since it diminishes the ability of the polymorphonuclear cells to phagocytize microorganisms (Ward and Spears, 1998). The use of Cu from the diet is greatly inhibited by the consumption of antagonists such as iron (Fe), sulfur (S), molybdenum (Mo), and zinc (Zn) (Ward *et al.*, 1997).

Se is part of the glutathione peroxidase (GSH-Px), an enzyme that prevents damage to cellular membranes and participates in the immune response of animals. This element is an antioxidant and its deficiency is responsible for alterations in the function of the immune system, the metabolism of the thyroid gland of the reproductive system, and the modulation of inflammatory processes. Its deficiency diminishes the phagocytic capacity of polymorphonuclear cells (Lugton, 2004). Phillips and Humphryes (1987) and Lepper *et al.* (1989) have shown that there is a relationship between Paratuberculosis, Fe excess, and Cu deficiency in the soil. In the USA, there are areas rich in Fe and deficient in Se , indicating multiple interactions between the minerals mentioned above and Paratuberculosis (Ward and Perez, 2004).

Lugton (2004) and Downs *et al.* (2008) have shown a possible link between mycobacterioses and micronutrient deficiency. These authors also analyzed the possible effects of different micronutrients from the bovine diet on cell-mediated immunity and on the pathogenesis of bovine Paratuberculosis and bovine Tuberculosis, respectively.

The objective of this work was to study the relationship between the presence of Paratuberculosis in two beef cattle herds and mineral imbalances in animals, soil, pasture or water.

Materials and Methods

Animals

Bovine older than 3 years old without clinical symptoms of Paratuberculosis were selected from two beef herds, herd 1 (H1) and herd 2 (H2), from two different regions of the Province of Buenos Aires, Argentina. The group from H1 included 45 Shorthorn adult cows whereas that from H2 included 30 Aberdeen Angus and Hereford-crossed adult cows, grazing on natural or implanted pastures with grass and legumes *ad-libitum.* Additionally, one cow from H1 and one cow from H2 with clinical symptoms of Paratuberculosis (diarrhea and emaciation) were euthanized and feces were taken for culture and the lymph node and ileocecal valve were examined by gross pathology and culture.

Sample collection

Blood and fecal samples were taken from all animals to identify and quantify the disease and the level of minerals, respectively. Pasture and drinking water samples were obtained from different areas for the quantitative analysis of minerals.

Feces

Feces were taken from the rectum of each animal in sterile containers, refrigerated at 4 °C and processed the following day for culture examination of *Map*.

Blood

Blood was obtained by venipuncture and divided into three aliquots: a) serum for ELISA test, b) serum for quantification of minerals, and c) blood collected in a tube containing heparin to quantify hemoglobin and determine GSH–Px enzyme activity.

Pasture

Pasture samples were taken from each location where animals had previously grazed and then kept at 4 °C until processing for the quantitative analysis of minerals.

Drinking water

Water samples from the watershed and drinking troughs were taken in 500 mL plastic bottles and stored at 4 °C until processing for the quantitative analysis of minerals .

Laboratory examination

Culture examination for the presence of Map

Fecal samples (10 g) were decontaminated in 100 mL of a 0.75% hexadecylpyridinium chloride solution (Sigma, USA) in sterile bi-distilled water, and then stirred for 30 min at room temperature and allowed to settle. An aliquot of 40 mL of the supernatant was kept overnight at room temperature and then centrifuged at 2000 rpm for 15 min. The pellet was re-suspended in 1 mL of phosphate buffer saline (PBS) and this suspension was used as an inoculum for the bacteriological culture. Four drops were placed in tubes containing Herrolds culture medium either alone or supplemented with 2 mg/L of mycobactin J (Allied Monitor, Missouri, USA), and pyruvate (4.1 g/L) and a mixture of antibiotics (100 mu/mL nystatin, 2.0 mg/L amphothericin B, 100 μg/mL vancomycin, and 3.0 mg/mL nalidixic acid). The tubes were incubated at 37 °C for 16 weeks to identify the development of *Map* colony forming units (cfu) (Paolicchi *et al.*, 2003). The isolates of *Map* were processed by IS*900* PCR to confirm the presence of *Map* (Paolicchi *et al.*, 2003).

Serological testing

Sera were analyzed by indirect ELISA, with a slight modification of the technique described by Turnquist *et al.* (1991). The antigen used was a Paratuberculosis Protoplasmic Antigen (PPA-3 Allied Monitor, USA), a sterile-filtered, lyophilized protoplasmic cell extract of *Mycobacterium* sp, recommended for use in ELISA screening for the detection of antibodies produced against *Map*. We used 10 mg/mL of PPA-3 in carbonate buffer (pH 9.6) and this antigen was coated on plates (Immulon 1, USA) in a volume of 100 μL/well and incubated at 4 °C overnight. Sera were pre-treated with *M. phlei* to increase the specificity of the method. Each serum was diluted with PBS-TG (1:100) and 100 μL (in duplicate) was added to each well of the plate. Plates were incubated for 2 h at 15 °C and a 1:4000 dilution of the bovine antibody anti-IgG peroxidase (Sigma, USA) was added again and incubated for 1.5 h at 15 °C. Finally, 2.2-azino-di-ethyl-benzy-thiazoline sulfate (ABTS, Sigma), diluted in citrate buffer (pH 4.0) was added and read in a spectrophotometer (Multiskan Plus, Helsinsky, Finland) at a wavelength of 405 nm. Sensitivity and specificity (66% and 99%, respectively) defined by receiver operating characteristics (ROC) analysis using the MEDCALC program were determined previously (Paolicchi *et al.*, 2003). An animal was considered positive seroreactor when the optical density (OD) reached a value of 2.1 units or greater, suspect when the value was between 1.5 to 2.0 units and negative when the value was less than 1.4 units of OD.

Biochemical analysis in blood

The concentration of Se in blood was estimated by the activity of GSH-Px, which was spectrophotometrically measured using cumene hydroperoxide as substrate. Results are expressed as units of enzymatic activity/g of Hb (Berret and Herbet, 1979), and this was quantified with the colorimetric method, which measures the formation of hemoglobin cyanide in blood (Laboratories Wiener, Argentina). Iron, Cu and Zn were measured by atomic absorption spectrophotometry (A.A.S) according to Perkin Elmer Manual Lab (Perkin Elmer, 1982). Briefly, for each of the minerals, an aliquot of serum diluted in distilled water was taken. The sample to analyze Fe was previously treated with acetic acid to precipitate proteins. Then, all the samples to measure Cu, Zn and Fe were read by AAS.

Pasture analysis

Samples were obtained from each herd and approximately 3 kg of pasture previously identified, was cut simulated grazing height. Samples were subsequently mixed, quartered and dried in a forced air oven at 60 °C and then ground in a laboratory mill equipped with a mesh 20 and stored until analysis. In order to quantify the contents of Cu, Zn and Fe by A.A.S., grass samples were previously mixed with a mixture of nitric, sulfuric, and perchloric acid (3/2/2:v/v/v), and heat treated to total destruction of organic material (Fick *et al.*, 1979). Molybdenum was measured by colorimetric methods (Bingley, 1959).

Water analysis

The content of sulfates (SO_4) was quantified by a turbidimetric method (Cseh *et al.*, 1993). The contents of total dissolved salts were measured by the gravimetric method. The pH of the samples was measured using a pH-meter (Perkin-Elmer, USA). Normal values of minerals in pasture, water and serum were obtained from historical analysis from Biochemical Laboratories from Instituto Nacional Tecnologia Agropecuaria (INTA), Buenos Aires province, Argentina, for the last 10 years of mineral studies (S. Cseh, personal communication).

Statistical analysis

The optical density value in ELISA and the concentration of minerals in blood were analyzed by the "*t*" Student test, with 5% significance.

Results

Map isolation

A total of 15 *Map* strains were isolated from culture of feces and organs from all animals studied in H1 and H2, 13 *Map* strains from asymptomatic bovine (n = 75) and 2 *Map* strains from animals with clinical Paratuberculosis. The 13 *Map* isolates were recovered from asymptomatic animals of H1 (9 *Map* strains), and of H2 (4 *Map* strains) (Table 1). Based on the quantification of *Map* colonies per tube of the Herrold's medium, cows were classified as moderate (50 colonies) or high shedders (100 colonies or more), but there was no relation with results in ELISA. Two *Map* strains were isolated after 7 weeks of incubation on Herrold's medium from individual samples of feces and lymph nodes, ileocecal valve, and intestine from the two animals with clinical symptoms of Paratuberculosis, observing a high density of *Map* colonies (more than 100 colonies) on the culture tube. All *Map* isolates were confirmed positive by PCR in order to identify the IS*900* insertion sequence.

ELISA results

From all the asymptomatic animals from H1 and H2 analyzed, 13 (17.3%) were ELISA positive and 29 (38.7%) had suspect results in ELISA test (Table 2). From the animals with positive or suspect ELISA results (n = 42), *Map* was isolated from 31% of fecal samples cultured (Table 1).

Analysis of micronutrients from animals

From all the animals with positive results in ELISA test, 4 (30.8%) were deficient in Cu, and 7 animals (53.8%) were deficient in Se (Table 2), while all these animals were positive to *Map* in culture on Herrold's medium. From the animals with suspect results in ELISA, 14 (48.3%) were deficient in Cu, while 4 (13.8%) were deficient in Se (Table 2). Of the remaining 33 animals with negative results in ELISA, 16 (48.5%) were deficient in Cu and 4 (12.1%) were deficient in Se (Table 2). Additionally, the two animals with symptoms of Paratuberculosis and isolation of *Map* strains had normal levels of Cu but had the lowest enzymatic activity of GSH-Px of a total of animals studied (data no showed).

Analysis of micronutrients from pasture and water: The content of Mo and Fe in the pasture was high in both herds: 2.5 ppm and 1.134 ppm in H1, and 2.5 ppm and 2.019 ppm in H2, respectively. The Zn values in the pasture consumed by animals from both herds were either normal or moderately high, whereas the Cu levels were very low in the animals from H1 and normal in the animals from H2, presenting a Cu:Mo relation of 1.5 in H1 and of 5.2 in H2. The amount of total dissolved salts and SO_4 in the water samples was normal in both herds (Table 3).

Discussion

In Argentina there are no data about the relationship between the mineral status and the presence of Paratuberculosis in beef cattle. In this study, two cattle herds were studied for status of Paratuberculosis in serum and feces of animals and related to the concentration of oligoelements in blood, in drinking water and in pastures of each farm.

In the asymptomatic animals from H1, 9 *Map* strains were isolated from 45 individual samples (20.0%), while in the animals from H2 only 4 *Map* strains were isolated from 30 individual samples (13,3%). In both cases, the animals were asymptomatic to Paratuberculosis but showed positive results in serum by ELISA. The high values of OD observed in ELISA in the serum of animals from both herds were in relation with the positive isolation of *Map* in feces. *Map* recovery in feces from animals depends on the type of culture medium used and the decontamination procedure used. Due to the intermittent excretion of *Map* in feces, it is often necessary to take more than one sample, but in our work the *Map* isolation from one sampling from 20.0% of the animals without symptoms in H1 was considered a good result, since low bacterial recovery is frequent. The contamination was not present in the culture of feces from both herds, and culture samples from organs of the two euthanized animals with symptoms gave the best and most

Table 1 - Results from *Map* isolation, ELISA, Se and Cu quantification in blood from beef cattle of two herds with clinical history of Paratuberculosis.

		GSH-Px	(Se)	Cu	
		Low / Marginal	Normal	Low / Marginal	Normal
Positive animals for *Map* culture	ELISA positive and suspect n = 13 (100%)	11 (84.6%)	2 (15.4%)	9 (69.2%)	4 (30.8%)
Negative animals for *Map* culture	ELISA positive /suspect n = 29 (47.5%)	4 (13.8%)	25 (86.2%)	18 (62.0%)	11 (38.0%)
	ELISA negative n = 33 (52.2%)	-	33 (100%)	16 (48.5%)	17 (51.5%)
Total of animals		15 (20.0%)	60 (80.0%)	43 (57.3%)	32 (42.7%)

Table 2 - Results of ELISA of Paratuberculosis, blood mineral levels and contents of hemoglobin in animals.

		Cu (µg/mL)		Zn (µg/mL)	Fe (g/mL)	GSH- Px (UI GSH- Px/g Hb)	Hb (mg/100 mL)	Zn (µg/mL)
ELISA		Low / Marginal	Normal	Normal	Low / Marginal	Normal	Normal	Normal
Herd 1 (n = 45)	(+) = 8	NF	0.9 ± 0.31 (0.6-1.4) n = 8	1.6 ± 0.27 (1.3-2.0) n = 8	1.4 ± 0.17 (1.1-1.7) n = 8	22.3 ± 8.50 (19-32) n = 5	52.2 ± 14.35 (38-72) n = 3	12.2 ± 0.75 (11.6-13.8) n = 8
	(S) = 21	0.4 ± 0.12 (2.2-0.5) n = 6	0.8 ± 0.15 (0.6-1.0) n = 15	1.4 ± 0.39 (0.7-2.0) n = 21	1.3 ± 0.24 (0.9-1.9) n = 21	34 ± 0.71 (33-34) n = 3	56 ± 12.13 (42-88) n = 18	12 ± 1.24 (10-15.6) n = 21
	(-) = 16	0.5 ± 0.05 (0.4-0.5) n = 4	0.9 ± 0.18 (0.6-1.3) n = 12	1.3 ± 0.30 (0.7-2.1) n = 16	1.3 ± 0.21 (1.0-1.7) n = 16	32 ± 4.94 (28-35) n = 2	55 ± 13.78 (38-78) n = 14	12.4 ± 1.28 (10-14.4) n = 16
Herd 2 (n = 30)	(+) = 5	0.4 ± 0.11 (0.3-0.5) n = 4	0.7 n = 1	0.8 ± 0.13 (0.6-0.9) n = 5	1.3 ± 0.14 (1.1-1.4) n = 5	16 n = 2	49 ± 7.74 (40-58) n = 3	14 ± 1.00 (12.2-15.0) n = 5
	(S) = 8	0.4 ± 0.08 (0.3-0.5) n = 8	NF	1.0 ± 0.16 (0.8-1.4) n = 8	1.3 ± 0.24 (0.9-1.5) n = 8	33 n = 1	61 ± 18.59 (40-94) n = 7	14 ± 1.29 (11.3-15.6) n = 8
	(-) = 17	0.4 ± 0.11 (0.2-0.5) n = 12	0.7 ± 0.08 (0.6-0.8) n = 5	0.9 ± 0.12 (0.6-1.1) n = 17	1.2 ± 0.14 (0.9-1.5) n = 17	32 ± 1.41 (31-33) n = 2	62 ± 22.65 (40-120) n = 15	14 ± 1.98 (11.3-20.7) n = 17

The values show the mean ± standard deviation, range (*), and number of animals (n) in each herd. Reference values (Cseh S., personal communication from Biochemical Lab, INTA): Cu: 0.5-1.5 µg/mL; Zn: 0.5-1.5 µg/mL; Fe: 0.89-2.50 µg/mL; Se: > 30 UI GSH- Px/g hemoglobin; hemoglobin: 9.5-14 mg/100 mL; *NF*: Not found.

rapid results of colonies growth in Herrolds medium where *Map* was isolated as fast as 30 $\pm$ 2 days of culture. ELISA testing proved to be a useful screening tool to diagnose Paratuberculosis within a bovine herd without clinical symptoms. The results coincide with those found in the scarce literature on the interrelation between Paratuberculosis and mineral deficiencies in cattle (Lugton, 2004). It has been demonstrated that mineral deficiency predisposes to a decrease in the immune response of animals (Suttle and Jones, 1989). Escalation of Paratuberculosis from asymtomatic animals to a more clinical state marked by diarrhea and weight loss is thought to be caused by immune dysfunction (Stabel, 2010).

The greatest progression of Paratuberculosis may happen when animals have a very low activity of GSH-Px, as determined in the two animals with clinical symptoms, positive ELISA and *Map* isolation of feces. These lower levels of enzymatic activity in GSH-Px and Se deficiency in animals could make them less resistant to infectious diseases (Suttle and Jones, 1989) and cattle infected with *Map* isolates could thus manifest the clinical disease more easily. Selenium deficiency causes changes in the behavior of phagocytic cells of the immune system, diminishing the ability of neutrophils to phagocytize microorganisms (Boyne and Arthur, 1981). As for the activity of the GSH-Px enzyme, the rest of the animals in H1 showed no Se deficiency although animals that were ELISA positive represented the highest percentage of animals with a low activity of GSH-Px. This result could explain the tendency towards the relationship between the presence of disease and the low enzymatic activity (Grasso *et al.*, 1990; Miller *et al.*, 1993; Cao *et al.*, 1992). On the other hand, we did not observe relationship between *Map* infection and enzymatic activity of GSH-Px in the animals of H2.

We also found low levels of Cu and high values of Mo in the pasture grazed by animals in H1, with a Cu:Mo relationship of 1.5. These results could be the cause of a primary and secondary Cu deficiency in the animals. In contrast, the pasture grazed by animals in H2 showed an adequate relationship (Cu:Mo 5.2). The contents of both Mo and Cu in H2 were increased, so the Cu:Mo ratio indicates that the pasture is not risky for the animal. In contrast, the values of Cu in H1 were very low, which makes Mo concentrations interfere with the metabolism of Cu. The results obtained in animals with deficiencies in Cu levels did not show a major relationships with the animals positives for cultures of feces but did demonstrate an inadequate Cu:Mo ratio in the soil and pastures grazed by animals with *Map* isolates. In this work, 80% of the ELISA positive animals in H2 had marginal or deficient Cu values, but 100% of the ELISA suspect animals had low values of Cu, possibly due to the excess of Mo with normal Cu levels in pasture grounds by these animals.

Suttle and Jones (1989) demonstrated that high levels of Mo could cause a secondary Cu deficiency and low resis-

Table 3 - Contents of minerals (ppm) in the pasture and total dissolved salts (salinity), sulfates (SO_4) in mg/L and pH of water in two cattle herds with antecedents of Paratuberculosis.

		Pasture					Water		
	Samples	Cu	Mo	Fe	Zn	Cu:Mo relation	Salinity (mg/L)	SO_4 (mg/L)	pH
Herd 1	Lot AB	1.7	3.3	171	21	0.5	872	150	8.8
	Lot 21	6.2	2.7	189	26	2.3	858	170	8.1
	Lot 22	3.5	2.2	1059	26	1.6	1156	215	9.0
	Lot 23	3.0	1.8	3118	50	1.7	*NM*	*NM*	*NM*
	Mean	3.6	2.5	1134	31	1.5	962	178	8.6
Herd 2	Lot 1	12.2	4.4	751	24	2.8	108	25	7.3
	Lot 1-2	12. 0	1.2	3400	47	10	*NM*	*NM*	*NM*
	Lot 3-1	11.0	2.3	2137	52	4.8	*NM*	*NM*	*NM*
	Lot 3-2	6.8	2.1	1790	34	3.2	840	20	7.7
	Mean	10.5	2.5	2019	39	5.2	474	22.5	7.5

Reference values in the pastures and water (drinking troughs and watershed) in Buenos Aires province, Argentina (Cseh S., personal communication from Biochemical Lab, INTA): Cu: 5 ppm; Mo: 2 ppm; Fe: < 1000 ppm; Zn: 25 ppm; Cu/Mo: 4.0; Salinity: < 7000 mg/L; SO_4: 1000-1500 mg/L; pH: 6.8; *NM*: not made.

tance of animals to infections. Copper deficiency in cattle can cause the lowest activity of neutrophils to phagocytize microorganisms (Boyne and Arthur, 1981; Ward *et al.*, 1997; Ward and Spears, 1998). An excess of Zn in the diet could reduce Cu absorption and Cu concentration in plasma and the liver of animals, causing disease by diminishing immunity (Bremmer *et al.*, 1976). The function of the immunological system is maintained when there are symptoms of hypocuprosis, but is affected after a long period of deficiency of this mineral (Ward and Spears, 1998). However, in other species used for experimental investigation such as the mouse, Cu deficiency considerably diminishes the immune response immediately (Ward *et al.*, 1997). Copper deficiency due to the presence of antagonists such as Fe, Mo or SO_4, and Se deficiency could negatively affect the immunological system of the animals, and the capacity of a response to infection with *Map*.

There are evidences that implicate soil acidification, excess of Fe and Mo and marginal deficiencies in Cu and Se in the disease process in animals. A study of Paratuberculosis prevalence in cattle in Michigan (USA), found that an increase of 0.1 in soil pH was associated with a 5% decrease in the number of ELISA-positive animals (Johnson-Ifearulundu and Kannene, 1999).

Selenium deficiencies in animals affect the Fe-binding capacity of transferrin, resulting in increased cellular heme and Fe concentration (Bartfay, 2003). Besides, high quantities of Fe were found in the pasture grown in flooded areas, where the animals from H2 remained grazing for long periods. It has been mentioned that acid soils could favor the progression of Paratuberculosis, because Fe is available to be captured by *Map* (Ward and Perez, 2004), and this could be a predisposing condition for the development of this disease in H2. An increase in Fe content in the soil by 10 ppm was associated with a 4% increase in the number of ELISA-positive animals with Paratuberculosis. Because cattle frequently ingest soil along with the pasture, it has been suggested that the dietary intake of Fe could allow the clinical expression of Paratuberculosis (Johnson-Ifearulundu and Kannene, 1997). On the other hand, the Fe available in the soil has been proposed to enhance the survival or growth of *Map* in the environment and thereby improve the chances of transmission (Michel and Bastianello, 2000; Riviriego *et al.*, 2000). Fe is involved in oxygen transport and storage and is a cofactor of enzymes like catalases, and a deficiency in Fe decreases phagocyte function, lymphocyte proliferation and natural killer cell activity (Hulsewe *et al.*, 1999). In the rumen, Fe and S form a complex of iron sulfide which interacts with Cu in the abomasum, diminishing its availability and causing secondary Cu deficiency (Cabrera Torres *et al.*, 2009). In this work, we found that the concentration of SO_4 in the water in H2 was low, which would not cause interference with Cu metabolism.

The results obtained in the present work cannot determine whether deficiencies in microelements play a definitive role or how they cause a predisposition in the relationships of Paratuberculosis in beef cattle. However, we consider that the lowest Se levels found in animals with *Map* isolation but without clinical symptoms of Paratuberculosis (n = 13 animals) are relevant, because 84.6% of these animals were Se deficient. Moreover, the two animals with evident clinical symptoms of Paratuberculosis, were also found to be severely deficient in Se. This result may indicate a possible relationship between infection with *Map* and Se-deficiency. Selenium has a role in important biological processes and there are at least 30 known selenium-containing proteins including deiodinases, selenoprotein P and GSH-Px, with a role in preventing cell and membrane damage induced by reactive oxygen intermediates and organic hydroperoxides (Lugton, 2004).

The results of Se deficiencies in animals with Paratuberculosis could indicate that these interactions are capable of showing clinical or subclinical Paratuberculosis in beef cattle. For optimal function of the immunological system, the composition of the diet and the micronutrient required by the animal appear to be important (Engle 2007, Downs *et al.*, 2008) and it is possible that the clinical expression or immunological expression of Paratuberculosis or other mycobacterioses in the animal are more evident when micronutrient deficiencies are present.

Further epidemiological and experimental studies implicating excesses of Fe and deficiencies in Cu and Se and possibly other elements such as Zn, manganese and calcium in the clinical expression of Paratuberculosis and other mycobacterial diseases are necessary. Besides, more research directed at understanding the possible role of these micronutrients and their actual involvement in Paratuberculosis expression should be carried out.

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