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Research Article

Relationship between Sanitary Management and Co-Infection with *Salmonella enterica subsp. enterica* Sorovar Dublin and *Paracooperia nodulosa* in Buffalo Calves

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Abstract

Paracooperia nodulosa is an intestinal parasite of buffalo, commonly described in calves between four and six months of age. Inadequate sanitary management practices predispose calves to infection of parasitic, viral or bacterial gastrointestinal pathogens and increase mortality in the herd. Salmonellosis in buffalo calves is a disease characterized by severe intestinal lesions, intermittent and profuse diarrhea, and severe dehydration, with risk of sepsis. This work aims to relate deficiencies in hygiene and sanitary management with an outbreak of co-infection of Salmonella Dublin and *Paracooperia nodulosa* in buffalo calves from a dairy farm. Two calves were subjected to autopsy, where swabs were collected from different segments of the intestine, liver and spleen for microbiological analysis. Macroscopically, there were firm, multifocal to coalescent nodular lesions distributed in the wall of the intestine, predominantly in the jejunum and ileum. These lesions were characterized by mural granulomatous nodules associated with parasites (*Paracooperia nodulosa*). The imprecise anamnesis on the hygienic-sanitary management of the animals and the necropsy findings suggestive of salmonellosis, stimulated a technical visit to the property. At the site, rectal swabs were collected and faeces collected for complementary tests. The health situation of the dairy farm was precarious and the management adopted was inadequate. Changes in the infrastructure of the milking facilities and in the management of the animals were suggested. After six months, a new technical visit to the property was carried out without any improvement. The coproparasitological examination of the feces samples revealed numerous eggs of *Strongylida sp*. and *Strongyloides sp*. The bacterium *Salmonella spp*. was isolated from the intestinal swabs and after isolation was identified *Salmonella enterica subsp. enterica* serovar Dublin. It was concluded that endoparasitosis was an essential factor for the establishment of infection by Salmonella Dublin and that, possibly, the precarious conditions of hygiene of the facilities favored the spread and persistence of pathogens in the animals and in the environment. Poor health management is risk to public health.

Keywords: Endoparasite; Water Buffalo Enteritis; Salmonellosis; Public Health

Introduction

Water buffaloes are known for a greater resistance to some diseases when compared to bovines, however buffalo calves are more frequently affected by gastroenteritis than bovine calves, with mortality rates as high as 70% in water buffalo species [1]. This difference might be due to a greater susceptibility of water buffalo to gastroenteric pathogens, although it also may reflect the lack of appropriate management practices for this animal species (ALFANO, 2014). The majority of mortality rates in buffalos occurs in young animals within six months of age associated mainly with diarrhea [2]. The main etiological agents are enterotoxigenic *E. coli, Clostridium sp., Salmonella sp.* Rotavirus*, Eimeria sp. e Cryptosporidium sp.* [2].

Salmonella spp, alone or in combination with other microorganisms is responsible for huge economic losses in production and as zoonotic agent as well. The infection with Salmonella in buffaloes had already been studied in buffalo calves been responsible in 13% to 25% of the gastroenteritis in buffalo calves, with a mortality between 40-72% [1].

Infected cattle may excrete up to 10^8 CFU/g of feces and pollution of the environment is the main source of infection and subclinical carriers maintains the pathogen in the herd for a long time. Cattle that has discharge of Salmonella but show no clinical symptoms are known as active carriers. Those who gain immunity and have oral contact with the pathogen and subsequently pass them

in their feces without being infected are known as passive carriers. Latent carriers are the ones who has the pathogens in tissues and when submitted to stress, or other diseases return do disseminate the pathogen (DEMIRBILEK, 2018)

Female latent carriers after the stress of parturition return to spread Salmonella, hence the stillborn are highly susceptible to infection [3]. Clinical infected calves have hyperthermia, profuse diarrhea with mucus expelling and dehydration (BORRIELLO et al. 2012), upon the release of enterotoxins when abdominal pain, apathy, anorexia, tachypnea and subsequent death within four to seven days can occur [1].

Paracooperia nodulosa a tricostrongilide specific for buffaloes, parasite the intestine walls forming nodules and is considered most pathogenic parasite in the small intestine of buffaloes [4]. Infection pressure of this nematode is directly related to environmental conditions, since the development and migration of the larvae in pastures is facilitated by the humidity in the rainy season [5]. Despite the large literature on parasite control in buffaloes, parasitic infestation remains a problem in young buffalo calves and results in high mortality rates [5]. Moreover, the parasite causes severe immunosuppression in the host and predisposes it to complications, such as secondary bacterial infection [6].

Epidemiologically the introduction of new animals, the reuse of stercum as fertilizer, changes in management, presence of wildlife, high rates of density and the mixing of different categories are important factors to increase infection [7]. Taking this in account the sanity of the herd and installations must be restrictedly controlled. Vaccination of the herd and good nutrition are minimal prophylactical actions to control and prevent infections [8,9].

The present study aims to describe the anatomopathological findings of a coinfection of Salmonella Dublin and *Paracooperia nodulosa* in bubaline calves and relate then to the management practices of the herd and the property.

Materials and Methods

The study was carried out between May and December 2016 in a dairy farm located in the State of São Paulo, Brazil, with 800 heads, from which 220 were females on location. Epidemiological data were obtained from the owner and workers. In May 2016, two corpses of buffalo calves, with four months of age, were sent to necropsy in the Department of Veterinary Pathology (DPVe, FCAV-UNESP), Jaboticabal, São Paulo State, Brazil. Fecal samples from other seven calves were sent as well. In June 2016, an ill calf was admitted in the "Governador Laudo Natel" Veterinary Hospital of the same institution, the animal showed hyperthermia, dehydration and profuse diarrhea and died three days after admission and was also necropsied.

Tissue samples collected at the necropsy of the three buffalo calves were fixed in 10% buffered formalin, embedded in paraffin, sliced with 5 µm thick and stained with hematoxylin-eosin (HE). During the necropsy, swabs from different segments of the intestine, from the cut surface of the liver and spleen for microbiological examination. A visit was made to the property in June 2016. At that time, there were 43 ill animals, of which 36 were calves up to four months of age and seven were adults. Fecal samples and rectal swabs were taken for parasitological and microbiological examinations, respectively. The swab samples were stored in a sterile flask with saline solution and stored under refrigeration until microbiological analysis for *Salmonella* spp [10]. After the isolation of the bacteria, the sample was sent to the Adolfo Lutz Institute (São Paulo, SP, Brazil) to identify the serovar where complementary tests (PCR and serology with O9 and Hgm sera) were made. All fecal samples were sent to the Animal Parasite Diseases Department of the Department of Preventive Veterinary Medicine and Animal Reproduction (MVPRA, FCAV-UNESP) for coproparasitological analysis using the modified Gordon and Whitlock [11] method.

After the conclusion of the clinical treatment, a second visit to the property was carried out. The objective was to verify, besides the health of the herd, if the suggestions offered in the sanitary management of the farm had been complied with.

Results

A mortality rate of 43,8% of calves born in the season was related by the owner in between the period of the study. However, a lack of precise information about the clinical story of the calves is patent. The calves were found dead by the morning after the recognition of clinical signs by the workers, that were apathy and bilateral ocular secretion. The sick animals were treated with a wide range of therapies including enrofloxacin, toltrazuril and diminazene diaceturate, all of which with different days of treatment not precisely related by the farm workers. The animals did not receive fluid therapy, despite showing signs of dehydration. It was also reported that there was intermittent diarrhea in the animals, but without prior separation of the diseased animals from the healthy ones.

The main findings at the necropsy of the three calves were pale ocular, oral, penile and vaginal mucosa, mild to moderate dehydration, presence of yellowish and soft stools adhered to the perineum. Cutting the skin revealed uncoagulated blood flowing from blood vessels, pale musculature, and gelatinous subcutaneous tissue (edema). In the in situ evaluation of the abdominal cavity, there were numerous visible multifocal nodulations in the small intestine wall, the mesenteric lymph nodes were markedly enlarged and the mesenteric vessels were markedly engorged with blood (Figures 1A, 1B and 1C).

In the serosa of all segments of the small intestine were whitish and firm nodules, which were about 0.5 cm in diameter (Figure 1A, 1B and 1C). In the intestinal lumen, the contents were greenish-

yellow and varied from fluid to mucoid (Figure 1D). At the cutting of the nodulations, a grayish central area delimited by a homogeneous whitish area. In the other organs, no macroscopic changes

Figure 1: Gross morbid of 4-month-old Murray buffalo calf. (A) Note the mesenteric lymph nodes enlarged in the abdominal cavity (arrows) and white whitish nodules on the wall of the small intestine (arrowhead) and mesenteric vessels engorged by blood (yellow arrowhead) are also evident. (B) The nodular aspect of the small intestinal wall is similar to described in figure A. (C) The visible nodules also in the intestinal mucosa, characterizing a mural process (*). Note the enlarged mesenteric lymph nodes (arrow). (D) Appearance of the faeces greenish and pasty in segment of the small intestine (arrow).

Microscopic examination revealed intestines with a marked inflammatory infiltrate composed predominantly of macrophages with uncolored and distended (foamy) cytoplasm, as well as multinucleated cells that extended from the mucosa to the serosa (Figure 2). In several segments this infiltrate invaded the muscular layer (Figure 2C) and the myenteric plexus (Figure 2D), dissociating the cells of these tissues. Peyer's plaques were markedly reactive, but with severe infiltration of foamy macrophages. In other segments of the intestine, multiple nodular formations were distributed throughout the submucosal and serous extension. In the center of the granulomatous lesion of these nodules' nematodes were observed (Figure 2E, 2F and 2G), surrounded by exuberant inflammatory infiltrate composed of macrophages with foamy cytoplasm, which gave a xanthomatous aspect to the lesion. The mesenteric lymph nodes had intense cortical lymphoid rarefaction, multifocal areas of necrosis and a marked infiltration of foamy macrophages, which distorted the lymph node architecture (Figure 2H).

The lungs were diffusely congested, with moderate edema, discrete foci of necrosis and areas of subpleural atelectasis, thickening of the interalveolar septa, due to the presence of intense inflammatory infiltrate composed of lymphocytes, foamy macrophages and

Figure 2: Photomicrography of the small intestine of buffalo calf. (A) Acute macrophage infiltrate with foamy cytoplasm extending from the submucosa (arrows) to the muscular layer of the intestine (Obj. Lens 10x). (B) Detail of the invasion of the muscular layer (*) by the foamy macrophages (arrows, Obj. 4x). (C) Detail of B, with macrophages (arrow) dissociating the smooth muscle fibers (*) from the intestine (Obj. Lens 20x). (D) The macrophage infiltrate reaches the myenteric plexus (*, arrows, obj. Lens 40x). (E) Presence of nematodes in the submucosa of the small intestine, circumscribed by macrophagic inflammatory infiltrate (arrows, Obj. Lens 4x.). (F / G) Detail of intralesional nematoda (Paracooperia nodulosa) in duodenal submucosa (Obj. Lens 20x). (H) Mesenteric lymph node with intense lymphoid rarefaction (*, Obj. Lens 10x). In particular, the presence of foamy macrophages (*) interspersed in the lymphoid tissue (Obj. Lens 40x). Hematoxylin and Eosin.

areas of marked hemorrhage. The spleen had moderate hemosiderosis in red pulp and lymphoid rarefaction in white pulp. The liver had diffuse congestion, mild hemosiderosis, swollen hepatocytes, mild proliferation of lymphocytic infiltrates and lymphocytic infiltrates, and foamy macrophages on the sinusoids. Kidneys were with hypercellular and enlarged glomeruli and with focal fibrosis of Bowman's capsule.

The *Salmonella bacterium* was isolated from the samples collected during necropsy and rectum swabs samples taken at the property. After isolation, the sample of *Salmonella* spp. was identified as *Salmonella enterica subsp. enterica* serovar Dublin.

The hygienic-sanitary conditions of the buffalo farm facilities and the sanitary management of the animals were evaluated through an on-site visit in which several problems were identified. The milking of the buffaloes was performed once a day, with the calves being used to stimulate the milk ejection, without the adoption of pre and post-dipping, no tests for prevention and control of mastitis, such strip cup test or California Mastitis test (CMT). In the milking parlor, sanitary conditions were very poor (Figure 3), with no water drainage system from the milking pit, which was not cemented, as well as accumulation of organic matter (feces, urine) on the floor, teat-cups and fomites (Figures 3B, 3C). The owner stated cleaning the floor of the milking parlor with chlorine diluted in water. However, it was found that this product was spilled on the floor containing organic waste (feces) with a hose. In the end, a portion of calcium oxide was thrown over the dirt on the floor.

Figure 3: Hygienic-sanitary conditions of buffalo farm facilities. (A) Tandem milking parlor (Indian row), mechanized milking and pit with uncemented floor. Notice the accumulation of feces on the floor (*). (B) Poorly sanitized milking units with faeces (arrows). (C) Trough with water accumulation. (D) Note organic matter adhered to the hose (*). (E / F) Pit with very poor hygiene conditions.

On regard the sanitary management of the herd, there was no standardized schedule of vaccination for colibacillosis, leptospirosis, clostridiosis, infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea (BVD), in addition to mandatory Brazilian vaccines (brucellosis, tuberculosis and rabies). The schedule of verminosis control was also not carried out, being the treatment against parasitosis instituted without technical criterion or guidance of a veterinary.

During the visit, improvements were suggested in the milking parlor, such as cementing the milking parlor and creating a drainage system for effluents. Regarding the animals, it was recommended to install a corridor with water slides under pressure at the entrance of the milking anteater, which would allow the buffaloes to cool and simultaneously eliminate dirt and flies from the skin. It was also suggested to avoid the accumulation of mud and feces in the housing area, to avoid contamination of the udder. The importance of pre and post-dipping of the teats with disinfectant solution was emphasized to reduce risks of mastitis development and milk contamination, and that, once the milking was completed, the milking apparatus should be cleaned and the washing of the milking system should be done regularly. Hygienization of the milking parlor should be done with detergent, to remove dirt and disinfect with suitable sanitizing agent, as a chlorinated alkaline detergent for cleaning walls and floors.

The necessity of a qualified technical responsible to guarantee both the handling and the adequate sanitary status of the animals was emphasized, the importance of a vaccination schedule, the failures of the indiscriminate use of medicines and the importance of the removal of the dirt before disinfecting the milking parlor and its equipment were reported. After the result of salmonellosis, another contact was made with the owner informing him about the disease and alerting about its zoonotic nature. At this time, the recommendations stated above were reaffirmed.

During the second visit to the farm (December 2016), the farm owner reported having followed the recommendations made during the first visit regarding the clinical treatment of the animals and sanitary management of the facilities. Nevertheless it was observed that few alterations were made, with persistency of deficiencies, water and feces were noticed in the milking pit and the facilities weren't changed, since most of the milking parlor was composed of wood, with gaps between the boards, which allowed the entry of dust and the accumulation of dirt (Figure 4). The presence of dogs and cats was also observed in the milking environment. It was also suggested that hygienic-sanitary measures be adopted in relation to the environment, drugs, employees and animals. The importance of the hygiene of the milk workers (clean clothes and good hygiene) and the need to clean and disinfect the floor and equipment, with adequate removal of dirt and organic waste, before the application of disinfectant products were emphasized. In addition, greater attention was paid to the health of the animal (adults and calves) in the management before and after milking, the importance of separating diseased animals from healthy ones or of those being treated was evidenced, and it was also recommended to avoid the indiscriminate use of medicines.

Figure 4: Hygienic-sanitary conditions of the environment adjacent to the milking parlor. (A / B) Feces accumulation in the pre-milking period, where the buffaloes are to be milked. (C / D) Feces observed in the same place, after the end of milking. Notice a whitish powder (lime) thrown on the floor with stool (*).

The implemented treatment was with trimethoprim sulfamethoxazole, in addition to replenishment of fluids to the dehydrated animals. The antiparasitic treatment was with albendazole.

Discussion

Histopathological analysis showed that buffalo calves were affected by a severe mural granulomatous enteritis associated with the presence of intralesional nematodes (*Paracooperia nodulosa*), similar to that observed by Bastianetto and Leite [4]. The animals also had severe diffuse granulomatous lymphadenitis in mesenteric lymph nodes, marked lymphoid atrophy in lymph nodes and spleen, as well as interstitial encephalic edema, membranoproliferative glomerulonephritis, severe diffuse chronic interstitial pneumonia, which led to death due to respiratory insufficiency. A possible framework of immunosuppression was observed by the lymphoid rarefaction observed in lymphoid organs, such as lymph nodes and spleen.

The granulomatous enteritis associated with *Paracooperia nodulosa* and lymphadenitis were related to the presence of Salmonella Dublin. In many cases of salmonellosis, the inflammatory response is limited to the intestine, without the systemic spread of infection, but Kamal (2014) reports that the pathogenicity of *Salmonella* spp. is related to its ability to invade the host cell and resist digestion by phagocytes and destruction by the complement system. The survival of S. Dublin in the interior of macrophages is considered an essential component in determining the pathogenicity of this bacterium [12].

In cases where the immune response is not able to limit salmonella to the gastrointestinal tract, pneumonia, meningitis, polyarthritis, osteitis, among other complications are described [12,13]. The immunity developed by the host against parasites located in the intestine is inferior to that developed by these animals against the intracellular parasites [14]. Thus, it is suggested that in the buffalo calves of this case report, the immunosuppression induced by the intestinal parasite (*Paracooperia nodulosa*) associated with precarious animal health may have contributed to the systemic spread of S. Dublin.

Salmonellosis is one of the most common causes of neonatal diarrhea in cattle and buffaloes [1]. In the buffaloes of this study, the presence of foamy macrophages as described in the intestine and mesenteric lymph nodes was verified in the lung and liver, suggesting the systemic dissemination of salmonella, similar to that observed by Silva *et al.* [15], in calves experimentally infected with Salmonella Dublin.

The nematode *Paracooperia nodulosa*, observed in the small intestine of buffaloes, was diagnosed based on the location and morphological aspects of the parasite. According to Bastianetto and Leite [4], *Paracooperia nodulosa* is a species-specific nematode of buffaloes, responsible for the formation of extensive nodules in the intestinal mucosa, being considered the most pathogenic parasite in the small intestine of this animal species. The macroscopic lesions described in buffaloes [4] are similar to those observed in the animals of the present report, with nodules with a diameter of two to five millimeters with a diffuse distribution in the serosa and mucosa of the small intestine.

The poor hygienic condition of the farm facilities was a striking feature (Figures 3A - 3F, 4A - 4D), which together with inefficient sanitation of the facilities (incorrect application of chlorine and calcium oxide) favored the proliferation and dissemination of pathogens among animals and environmental contamination. It is known that action of disinfectants in the destruction or inactivation of microorganisms is not instantaneous. According to Both; Longanay; Avancini [16] the presence of organic matter significantly reduces the antibacterial efficacy of chlorine. The elimination of organic matter from the contact surfaces prior to the disinfection process is a primary condition for the use of chlorine as a disinfectant, since its oxidizing effect associated with organic matter results in the consumption of part of the disinfectant, reducing its effectiveness in destroying the microorganisms [17].

Fernandes and Furlaneto [18] demonstrated a satisfactory effect on the use of chlorine dioxide in the control of Salmonella spp, at pH higher than 10, leading to disorganization in the bacterial peptideoglycan structure. When the alkalization process occurs, OH- ions saponify the lipids that surround the bacteria externally, leading to the destruction of surface structures. In this property, the disinfection methods are empirical and, therefore, the efficacy of the use of disinfectants is clearly poor.

The calcium oxide is used as a sanitary barrier in the poultry industry and may be effective in reducing *Salmonella* spp., Depending on the bacterial concentration. Thus, treatments with calcium oxide at 300g/ m^2 should be efficient for the control of *Salmonella* spp., Because calcium oxide raises the pH, making the medium inhospitable to the bacteria [19,20]. The use of the chemical compound was at random, without a specific concentration and on the presence of organic matter. As the floor was not cemented, the elimination of organic matter and microorganisms present in the environment was even harder to accomplish.

Conclusion

Buffalo endoparasitosis was an essential factor for the establishment of Salmonella Dublin infection, as *Paracooperia nodulosa* induced an immunosuppressive condition the animals became more susceptible to a systemic infection. Coinfection of *Paracooperia nodulosa* associated with Salmonella Dublin led to death of buffalo calves. The precarious and poor conditions of hygiene of the facilities and the herd favored the dissemination and persistence of pathogens in the animals and in the environment. The zoonotic potential of the infection to the workers and to the milk produced was not accessed, but a risk to public health if the recommendations are not adopted is sound and proofed by the mortality rate of the animals and the economic losses already established.

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