Case Study

Neck Muscle Hemorrhage in an Alpine Kid Following Enterotoxemia: a New Necropsy Finding

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ABSTRACT

Enterotoxemia, also referred to as "Overeating disease" or "Pulpy kidney," is a condition caused by Clostridium perfringens type D. This condition poses significant economic challenges to the goat industry. The objective of the present report was to document a previously unreported necropsy finding, namely Neck Muscle Hemorrhage, observed in a deceased Alpine kid affected by enterotoxemia. The case in question involved a three-month-old Alpine kid that exhibited clinical signs indicative of acute enterotoxemia. A postmortem examination was promptly conducted to ascertain the underlying cause of death. Aseptic sampling of the small intestine, specifically the ileum contents, was performed during the postmortem examination. The samples were then examined bacteriologically. Additionally, a commercial enterotoxemia ELISA kit was utilized to identify the enterotoxins produced by C. perfringens, including Alpha, Beta, and Epsilon toxins, and to confirm the presence of bacteria in the obtained samples. During the postmortem examination, no overt external lesions were observed. However, necropsy revealed several remarkable gross lesions, including hemorrhage and hyperemia of the colonic mucosa and small intestine, pulmonary edema, hemothorax, hydropericardium, and neck muscle hemorrhage. The bacteriological investigation and ELISA assay results indicated the presence of alpha and epsilon enterotoxins in the intestinal contents, thereby confirming the detection of C. perfringens type D bacteria. Collectively, these findings provide substantial evidence that strongly links the observed lesions to enterotoxemia caused by C. perfringens type D infection in the examined alpine kids. Notably, the investigation identified a peculiar gross lesion—namely, hemorrhagic necrotizing lesions in the neck muscle—that has not been previously reported in goats and which is associated with enterotoxemia. The recognition of this distinctive lesion underscores its significance as a noteworthy manifestation of enterotoxemia in goats. The documentation of this lesion provides clinicians with valuable guidance during necropsy examinations, aiding in the recognition and diagnosis of cases of enterotoxaemia.

Keywords: Enterotoxemia, Alpine, Goat, Neck Muscle Hemorrhage, *Clostridium Perfringens.*

1. Introduction

Clostridium perfringens is the causative agent of intestinal infections that are often referred to as enterotoxaemia. This condition, known as "Overeating Disease" or "Pulpy Kidney," is primarily caused by the type D strains of C. perfringens. These infections have a notable economic impact on the goat industry because of the severe harm they cause. Both juveniles and adults within the goat population are susceptible to C. perfringens infections. It is noteworthy that C. perfringens is a resident microbe in the intestines of healthy animals. C. perfringens is an anaerobic bacterium. It is immobile, gram-positive, and rod-shaped. A salient attribute of C. perfringens is its capacity to form spores, a property that fosters its resilience and endurance in its environment. The presence of spores complicates the complete eradication of C. perfringens. Type D strains of C. perfringens are known to produce two primary toxins: epsilon (ETX) and alpha (CPA). Of these toxins, ETX is widely regarded as the primary virulence factor. The development of C. perfringens type D enterotoxemia in sheep and goats is primarily influenced by this toxin, which plays a pivotal role in altering the permeability of the vascular endothelium, consequently leading to the manifestation of the disease's characteristic signs (1-4). Following an increase in intestinal permeability, ETX is absorbed into the circulatory system with greater ease. Following its absorption into the circulatory system, the toxin binds to the microvascular endothelium of various tissues, including the lungs, heart, kidneys, and eyes. This binding can lead to damage and dysfunction of these organs, contributing to the overall impact of C. perfringens type D enterotoxemia (5-8). A multitude of factors may precipitate an escalation in C. perfringens population and the subsequent synthesis of toxins, culminating in enterotoxemia. These factors include a sudden overload of ingested carbohydrates, which occurs when the quantity of carbohydrates that can be digested in the diet surpasses the capacity of the intestinal mucosa to digest and absorb them. Furthermore, an abrupt alteration in feed type, such as a sudden increase in the consumption of grains, succulent pasture, high-protein diets, or rich grasses, can exacerbate the conditions that promote C. perfringens proliferation and toxin production (2, 5, 7, 9). Uzal et al. conducted a study in which an experimental model of enterotoxemia was established by intraduodenal inoculation of C. perfringens. The clinical signs observed in this model included dark green watery diarrhea with the presence of intestinal mucus and fibrin. Furthermore, the study noted the presence of respiratory distress and neurological signs, including recumbency, paddling, bleeding, convulsions, increased respiratory efforts, and opisthotonos. Gross lesions observed during necropsy included pulmonary edema, absence of cerebellar and brain herniation, hyperemia and edema in the gastrointestinal tract, necrotizing pseudomembranous colitis, and vasogenic edema (10). In a separate study by Ortega et al., ETX was detected in the intestines of 44 goats with type D enterotoxemia using ELISA. The most prevalent gross lesions identified during necropsy were hemorrhagic and/or necrotic inflammation in various areas of the intestinal tract, including enteritis, colitis, and typhlitis. Furthermore, the presence of liquid in body cavities, such as ascites, hydrothorax, and/or hydropericardium, as well as pulmonary congestion and edema, was evident (11). A presumptive diagnosis of type D enterotoxemia can be made by considering the history, clinical indicators, and gross postmortem lesions observed in the affected animals. While a presumptive diagnosis can be made based on these findings, laboratory confirmation and toxin detection are necessary for a definitive diagnosis. Ancillary tests, such as the examination of intestinal smears for the presence of gram-positive rods and the detection of glucose in urine, can support a diagnosis of enterotoxemia, but they cannot definitively confirm it. While the diagnosis is frequently made based on clinical criteria, numerous aspects of lesion occurrence in enterotoxemia remain to be fully elucidated. Further studies are needed to better understand the underlying mechanisms of this disease (5, 7). The primary objective of this study was to document a newly identified necropsy sign, referred to as "Neck Muscle Hemorrhage," observed during the diagnostic necropsy of a goat kid that died from enterotoxemia. The objective of this study is to contribute to the existing knowledge and diagnostic methods related to enterotoxemia in goat kids by documenting this novel necropsy sign.

2. Case Presentation

An outbreak of enterotoxemia has been observed among goats within an intensive breeding system. The signs of enterotoxemia exhibited by the affected goats included convulsions, high fever (41°C), vocalization, ventral abdominal pain (e.g., tucked abdomen and kyphosis), and rapid death. The goats in this herd had been vaccinated according to the guidelines set by the Iranian Veterinary Organization. The vaccination protocol included PPR, enterotoxemia, Rev 1, goat-pox, and FMD vaccines. The goats received a tetravalent vaccine against enterotoxemia, which targeted C. perfringens types D, C, and B, and Clostridium septicum (obtained from Razi Vaccine Research and Serum Institute, Iran). The mothers received subcutaneous injections of the vaccine in the neck region during the 12th and 16th weeks of pregnancy, whereas the children received three doses of the vaccine during the second, fourth, and eighth weeks after birth. In this herd, the last dose of the enterotoxemia vaccine was administered three weeks prior to the event under investigation. Immediately following the occurrence of the goats' demise, a postmortem examination was performed. A series of analyses were conducted on samples obtained from the small intestine, specifically the ileum content, through the use of aseptic sampling techniques. The samples were subjected to bacteriological, serological, and Gram staining analyses. The serological samples were meticulously transported to the laboratory in 1% chloroform, adhering to standardized protocols and ensuring a constant temperature

of 4°C. Following this, the intestinal specimens were diluted with endotoxin-tested distilled water at a 1:5 ratio. The samples were then subjected to centrifugation at 2000×g for 20 min at a temperature of 4°C. The resultant extracts were then extracted following centrifugation, filtered through 0.45-µm membrane filters, and subsequently preserved at -70 °C until subsequent use. The presence of C. perfringens and its enterotoxins (particularly alpha, beta, and epsilon toxins) in the samples was determined using a commercial Bio-X enterotoxemia ELISA reagent (Bio-X Diagnostics, Belgium). The instruments were utilized in accordance with the guidelines provided by the supplier to conduct the requisite analyses. This methodological approach enabled the identification and verification of enterotoxins and the presence of C. perfringens in the acquired samples. In summary, 100 µl of the diluted test samples, the positive control, and the negative control were added to designated wells on a 96-well microtitration plate to conduct the ELISA analysis. The plate was then subjected to an incubation period of one hour at a temperature of 21±3°C, with the lid securely fastened to ensure the integrity of the contents. Subsequent to the preliminary incubation period, the contents of the microplate were expelled, and the wells were rinsed thrice with 300 µL of washing solution. Thereafter, 100 µL of conjugate solution was added to each well, and the plate was returned to the incubator for an additional hour at a temperature of 21±3°C with the lid closed. Subsequent to the second round of washing, the microplates were returned to the incubator. Subsequently, 100 µL of chromogen solution was added to each well, followed by a 10-minute incubation at a temperature of 21°C+/-3°C in an illuminated environment, with the lid ajar. To halt the reaction, 50 µL of stop solution was added to each well after the final incubation. The optical densities (OD) of the samples were subsequently measured using an ELISA reader. To interpret the results, the requisite calculations were executed in accordance with the guidelines specified in the equipment manual. During the postmortem examination, no external lesions were observed on the bodies of the deceased goats. However, gross lesions were identified, including hemorrhage and hyperemia of the colonic mucosa and small intestine, as well as pulmonary edema, hemothorax, and hydropericardium. Subsequent examination of intestinal samples revealed the presence of large gram-positive rods. Subsequent biochemical analysis of these samples identified them as C. perfringens. The biochemical tests yielded negative results for Indole, Methyl Red, Urease, Oxidase, Catalase, and Mannitol fermentation, while exhibiting promising findings for Voges Proskauer, Citrate Utilization, Glucose and Sucrose Fermentation, Gelatin Liquification Test, Stormy Milk Test, Lecithenase Test, and H2S gas production. Furthermore, ELISA results indicated the presence of alpha and epsilon enterotoxins, as well as C. perfringens type D bacteria, in the intestinal contents. A notable finding during necropsy was the observation of a hemorrhagic necropsy lesion in the neck muscle of a three-month-old Alpine kid (Figure 1). This observation was unique and distinguished this particular case from the others, although it exhibited all the typical signs of acute enterotoxemia.

3. Discussion

In the present study, a case of acute enterotoxemia in an Alpine child was documented. The diagnosis of enterotoxemia was confirmed on the basis of clinical signs observed before death, necropsy findings, bacteriological analysis, and ELISA results. Notably, this particular case exhibited a unique necropsy finding: a hemorrhagic lesion in the neck muscle. This finding contributes to the existing body of knowledge concerning enterotoxemia and underscores the potential variations in lesion occurrence associated with this condition. After considering and ruling out other diseases that could potentially cause similar clinical signs and muscle hemorrhage, such as Blackleg, Black disease, and Tetanus, the diagnosis of enterotoxemia in the present case is further supported. These diseases were excluded based on the clinical, post-mortem, and laboratory findings specific to this case. It is noteworthy that necropsy lesions observed in cases of acute enterotoxemia can vary between sheep and goats. While both species may exhibit different necropsy lesions, sheep with type D enterotoxemia frequently display more pronounced neurological signs, and intestinal lesions are less commonly observed. This observation underscores the need for a meticulous and precise diagnosis, one that is informed by both clinical and pathological findings, as previously emphasized (12). The neurological signs associated with enterotoxemia in ovine subjects include blindness, aimless wandering, ataxia (uncoordinated movements), grinding teeth (bruxism), head pressing, nystagmus (involuntary eye movements), opisthotonos (the retracted curvature of the head and neck), and convulsions characterized by paddling movements. The severity of these neurological manifestations can serve as an indicator of the condition's intensity, aiding in the differentiation of enterotoxemia from other diseases with analogous clinical presentations (5). In instances of subacute and acute enterotoxaemia in ovine subjects, specific cerebral regions may manifest bilaterally symmetrical foci of malacia. These regions encompass the basal ganglia, thalamus, internal capsule, midbrain, medulla oblongata, and cerebellar peduncle. This neuropathological change is referred to as focal symmetric encephalomalacia (FSE) and is considered the most notable and recognizable lesion in the acute form of enterotoxemia caused by C. perfringens type D in sheep. In contrast, acute cases of enterotoxemia in goats and cattle rarely manifest this particular lesion. The occurrence of FSE in the brain is more commonly associated with acute forms of enterotoxaemia in sheep (3, 5, 13, 14). Further research is necessary to ascertain whether hemorrhage in the neck muscles can be considered a distinct gross lesion in goats. The distinctive observation of hemorrhagic necropsy lesions in the neck muscle of the Alpine kid in this study

Figure 1. Gross photograph of the hemorrhagic lesion in the neck muscle of the Alpine kid.

necessitates further investigation and validation. Conducting further studies and examining a larger sample size of goats with enterotoxemia could help establish the importance and frequency of this particular lesion in goats affected by this condition. A discussion of the mechanisms underlying the occurrence of these gross lesions is also warranted. In ruminants, including goats, the small intestine may contain C. perfringens type D bacteria in low concentrations, and the production of epsilon toxin (ETX) is typically restricted. However, a disruption in the microbial balance within the intestine can occur, resulting in the rapid multiplication of saccharolytic bacteria and the subsequent production of substantial amounts of ETX. Consequently, when ETX is produced in high concentrations within the gut lumen, a sufficient quantity of the toxin can enter the bloodstream. The disruption of the gut microbial balance can be triggered by various factors, including dietary modifications, stress, or the presence of other concurrent illnesses. The disturbance of this balance enables the proliferation of C. perfringens type D bacteria, leading to the production of elevated levels of ETX and the subsequent development of enterotoxemia. The toxins produced by these bacteria can then cause damage to various organs and tissues, resulting in the observed gross lesions, including the hemorrhagic necropsy lesion in the neck muscle observed in the Alpine kid in the present report (5). ETX is classified as a member of the aerolysin class of pore-forming toxins. ETX, a toxin produced by C. perfringens type D bacteria, has the capacity to bind to specific receptors located on endothelial cells. The binding of ETX to these receptors initiates a sequence of events that ultimately results in endothelial injury (5). The toxin oligomerizes into a heptameric pre-pore via lipid rafts and caveolins, generating an active pore on the host cell surface. The outcome of this process is the formation of capillaries, resulting in a rapid decrease in cytoplasmic K+ levels and subsequent introduction of Na+ and Cl- into the cell, leading to cell necrosis. The endothelial cells, which play a pivotal role in maintaining the structural integrity and functional capacity of blood vessels, are particularly vulnerable to impairment. This impairment can lead to a deterioration of the blood-brain barrier and an increase in blood vessel permeability. ETX-induced endothelial injury can result in a range of pathological alterations, such as necrosis, edema, and hemorrhage, all of which contribute to the observed gross lesions. The capacity of the toxin to compromise the structural and functional integrity of endothelial cells is a pivotal factor in the pathogenesis of enterotoxemia and subsequent tissue damage (15-17). Furthermore, the potential risks associated with ETX extend to the realm of zoonotics and bioterrorism. Botulism and tetanus are the two clostridial toxins with the highest potency, followed by ETX (1, 18). Sheep, goats, cattle, mice, and rats have been utilized in research endeavors aimed at elucidating the effects of intravenous ETX. The administration of toxins has been observed to induce vascular permeability in numerous tissues (19). According to previous studies, circulating ETX localizes and accumulates in the brain. The blood-retinal barrier (BRB) exhibits notable similarities with the blood-brain barrier

(BBB) in critical aspects. In acutely intoxicated rats, it has been demonstrated that ETX compromises the BRB in a manner analogous to the BBB. The observed effects of ETX on the eye include vascular disruption and severe endothelial damage, which is analogous to the effects observed in the cerebral endothelial cells of sheep and mice exposed to ETX (5, 13, 16, 20). The hemorrhage observed in the neck muscle, as described in the present report, could potentially be one of the effects of circulating toxins in the blood of goats. In contrast to sheep, which experience severe toxemia and fewer digestive disturbances, goats affected by enterotoxemia often exhibit more pronounced intestinal complications, including diarrhea, which can be hemorrhagic, severe abdominal discomfort, and hyperemic lesions. The enteric lesions observed in goats, manifesting in the subacute or acute stages of the disease, are likely attributable to the localized toxic action of ETX (5). One hypothesis suggests that the severity of enteric lesions in goats may be attributed to the slower intestinal absorption of ETX into the bloodstream compared to sheep. The hypothesis is that the absorption of ETX from the intestine into the bloodstream in goats is slower than in sheep (11, 21). However, it is imperative to acknowledge that this hypothesis necessitates further investigation and scientific validation. The precise mechanisms underlying the observed variations in enteric lesions between goats and sheep with enterotoxemia remain to be fully elucidated and may encompass a range of factors beyond the rate of ETX absorption (5). A notable distinction in the prevalence of lesions between affected goats and sheep is the occurrence of hemorrhage in the neck muscle. In a study conducted by Thedford and Johnson, the prevalence of infectious diseases in new-world camelids (e.g., guanaco, vicuña, llama, and alpaca) in the United States was examined in relation to other domestic animal species. Enterotoxemia caused by C. perfringens was identified as a prominent disease. The study documented two cases of type D enterotoxemia, manifesting in adult llamas. During necropsy examinations of these llamas, several notable lesions were observed. These included pulmonary congestion, petechial bleeding, and ecchymosis (bruising) of the lower neck and axillary muscles. Pericardial effusion containing fibrin clots was also observed. Notably, this study is the sole one to date that has specifically mentioned the presence of hemorrhagic lesions in the neck muscle in cases of type D enterotoxemia in llamas (22). The diagnosis of enterotoxemia is chiefly dependent on three factors: the animal's medical history, the manifestation of clinical symptoms, and the abrupt demise of the animal. However, the isolation of Gram-positive C. perfringens rods from the intestine alone was inadequate for confirming the diagnosis. Its diagnostic utility is limited when it comes to enterotoxemia in any species, as healthy animals commonly harbor microorganisms within their intestinal tracts. Conclusive evidence of enterotoxemia is best obtained through the identification of enterotoxins and necropsy of lesions (1, 3, 23, 24). Numerous studies have employed ELISA to detect enterotoxemia, specifically epsilon toxin, owing to its superior sensitivity and specificity. A variety of techniques, including counterimmunoelectrophoresis, monoclonal capture ELISA, polyclonal capture enzyme-linked immunosorbent assay, and mouse neutralization test, can be employed to detect ETX in the intestinal contents of sheep and goats. It is imperative to acknowledge that while bacteriological analysis and ELISA can offer significant insights into the diagnosis of enterotoxemia, they should be utilized in conjunction with clinical evaluations. This is due to the fact that the results of these methods have been discordant in some cases (1). ELISA is an adequate method for detecting epsilon toxins in intestinal contents. A substantial body of research has utilized ELISA due to its noteworthy sensitivity and specificity for the detection of enterotoxemia, particularly epsilon toxin (1, 25). A study employing the intestinal content epsilon toxin assay using Sandwich ELISA method reported exceptionally high sensitivity and specificity rates of 94.6% and 97.4%, respectively (26). A comparative study has investigated the efficacy of different body fluids and C. perfringens type D epsilon toxin detection methods in sheep and goats. Among these methods, polyclonal capture ELISA (PC-ElISA) has emerged as the most sensitive, irrespective of fluid type, in contrast to myoclonic capture ELISA, counterimmunoelectrophoresis, and MNT (25). Furthermore, the presence of C. perfringens type D epsilon toxin has been identified in the small and large intestine contents of goats with type D enterotoxemia via capture ELISA, which utilizes monoclonal antibodies as the detection method (21). The presence of hemorrhage in the neck muscles, as observed in the present study, can be considered a rare finding associated with acute enterotoxemia in goats. It is noteworthy that during necropsies, the neck muscles are not systematically examined. Consequently, the recognition and documentation of this lesion in the present report can offer substantial assistance to clinicians in establishing a definitive diagnosis of enterotoxemia based on necropsy findings.

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Authors' Contribution

Investigation of Concept and Design: H. E.
Acquiring information: H. E. and S. M. J.
Data interpretation and analysis: S.M.J.
Drafting the manuscript: S.M.J.
Strict revision of the manuscript in light of its significant intellectual merits: E.
Material, technical, and administrative assistance: H. E.

Ethics
In composing the originally submitted article, the authors affirm that they adhered to all ethical norms.

Conflict of Interest
The authors have declared that they have no conflicts of interest.

Data Availability
The data produced and/or analyzed throughout the present investigation are available upon request from the corresponding author.

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