Enzootic Bovine Hematuria

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Preface

Pocket Veterinary Medicine is a book series edited by Isabel Pires and Filipe Silva.

Being veterinarians, trained in pathology (IP) and clinical (FS), the editors aimed that each book could address the disease by seeking molecular, cellular, and organic changes to understand clinical signs and treatments.

Destined to veterinary medicine students and professionals, Pocket Veterinary Medicine intended to be a set of small books, easy to consult, and accessible to all. More than an exhaustive review of each topic, the book will approach a different subject in a methodical, simple and practical way.

We hope you like it and can be useful to you.

Contents

Introduction

Enzootic bovine hematuria (EBH) is a serious condition that mainly affects cattle from 4 to 12-year-old managed in extensive systems based on pasture. Calves are usually not affected. There is no evidence of breed or sexual predisposition for the development of EBH (Seifi *et al.*, 1995; Nielson & Moulton, 1990).

This syndrome is clinically characterized by the presence of hematuria, which can occur intermittently or continuously, accompanied by anemia (Divers *et al*., 2018).

Although BEH presented a worldwide distribution with variable prevalence, it has been mainly reported in tropical regions with less arable or non-cultivated lands (Vetter, 2009).

Since no curative treatment is currently available for BEH, affected cattle displayed a decrease in its performance, mainly characterized by emaciation, weight loss, and decrease milk yield (Giles & Andrews, 2008).

This syndrome is the clinical expression of tumors of epithelial and vascular origin present in the urinary bladder. The presence of the chemical compound, called Ptaquiloside, in bracken fern (*Pteridium* spp.), has been described as the main cause of the disease and the neoplastic lesions result from the carcinogenic property of this substance (Sharma *et al*., 2013). However, evidence suggests that urinary bladder neoplasia is enhanced with concomitant bovine papillomavirus-2 (BPV-2) infection in cattle (Resendes *et al*., 2011).

Etiology

Pteridium aquilum is the main fern responsible for the development of BEH, but other species of the genus *Pteridium* are also involved such as *Pteridium arachnoideum*, *Pteridium caudatum*, *Pteridium esculentum* and *Pteridium revolutum* (Vetter, 2009; Micheloud *et al*., 2017). Moreover, other plants belonged to other genus such as *Pteris deflexa* and *Pteris plumula,* rock fern (*Cheilanthes sieberi,* Australia), or *Onychium contiguum (*India), (McKenzie, 1978; Dawra *et al*., 2001; Micheloud *et al*., 2017) may also be implicated.

However, other factors such as nutritional deficiencies, mineral deficiencies in the soil, free-grazing management, and viruses (bovine papillomavirus) have been also associated with BEH (Bringuier-PP and Jean-Blain, 1987; Campo, 1997).

Pteridium aquilinum

Morphological description

Ferns are plants that do not produce flowers or seeds and are mostly herbaceous. The stems are known as rhizomes, because they grow horizontally above and below the soil surface however, there are also erect and climbers; most are covered by scales, but some are covered by hairs. The scales are generally lanceolate and sometimes orbicular. The hairs can be unicellular or multicellular, they are usually simple although some species are found with starry or ctenitoid hairs. Fern leaves known as fronds, are monomorphic in most species, however, they can be dimorphic in certain species. The sheets are described as simple or pinnate but are generally several times divided with a pinnate division pattern (Lee & Shin, 2011).

Geographic Distribution

Pteridium aquilium (figure 1) is native from Gilan province, Manzadaran, and Golestan, in Iran (Tourchi-Roudsari, 2014). Nowadays, *Pteridium aquilinum* is a cosmopolitan species with worldwide distribution except polar desert regions (Fernandes & Fernandes, 1983; Nunes & Castro, 1991; Durão *et al*., 1995). It is mainly located in acidic forest soils, warm dark areas, woods, and roadsides (Marrs & Watt, 2006; Tourchi-Roudsari, 2014).

Fig 1: *Pteridium aquilium*

There are two subspecies (Table 1) that include several varieties according to the geographic area.

Table 1: *Pteridium aquilinum* subspecies and varieties

Subspecies	**Aquilinum**	**Caudatum**
Varieties	Aquilinum Africanum Pseudocaudatum Latiusculum Wightianum Pubescens Decompositum Feci	Caudatum Arachnoideum Esculentum Yarrabense

The worldwide distribution of *Pteridium aquilium* is justified, in part, by its adaptive capacity to the environmental conditions. It is an extremely opportunistic plant that tries to maintain its dominance. Ferns presented some survival mechanisms such as synthesis of substances that once incorporated in the soil difficult the appearance and development of other plants or production of substances that act as a repellent of predators (Cooper, 1976; Gliessman, 1976; Fernandes & Fernandes, 1983). Also, bracken ferns can grow and adapt to a wide environmental conditions with scarce water requirements (Tourchi-Roudsari, 2014).

In Europe, there are mainly *Pteridium aquilinum* subs. *aquilinum*, *aquilinum* variety (Page, 1976).

In Portugal, bracken fern is found in acid and not too drylands across the country, at altitudes ranging from sea level to about 1500m in the Serra da Estrela. In Azores islands, its presence is uncommon above 1100 m of altitude (Franco & Afonso, 1982; Fernandes & Fernandes, 1983).

The expansion of *Pteridium aquilinum* worldwide represents a real invasive plague. Therefore, intrinsic characteristics such as high reproductive potential or propagules with long viability as well as external factors like human activity, intensive crops, or fires among others are risk factors of its expansion (Fernandes & Fernandes, 1983).

Toxic agents in Bracken

Bracken-fern contains some toxic constituents that vary according to the variety as ptaquiloside, pterosin, shikimic acid, thiaminase, and quercetin.

Ptaquiloside is considered a powerful toxic compound and also a strong carcinogen. It is responsible for the innumerous biological effects of the fern, such as bright blindness in sheep, mutagenicity, genotoxicity, and carcinogenicity in humans (Yamada *et al*., 2007).

Pterosin is widely found in ferns and is associated with *in vitro* cytotoxicity (Chen *et al*., 2008; Tourchi-Roudsari, 2014).

Shikimic acid might act as a carcinogenic stimulating factor although its action mechanisms are currently unknown (Jones *et al*., 1983; Tourchi-Roudsari, 2014).

Thiaminase is present in all parts of the fern. Its action mechanisms lead to the destruction of thiamine and subsequently to various health problems, such as beriberi in animals fed with bracken fern (Nielsen & Moulton, 1990).

Quercetin has pro-apoptotic effect that leads to cell damage and cytotoxicity. However, this effect has only observed in vitro studies (Tourchi-Roudsari, 2014).

Toxicity

Since bracken-fern is a poisonous plant for livestock, its toxic effect may vary regarding the amount ingested, time of exposition, season, or part of the plant as well as the animal species. Hence, horses presented anorexia and ataxia. In sheep, neuroepithelial degeneration of the retina leading to bright blindness has been described (Ugochukwu, 2018). In cattle, bracken poisoning also causes depression of hematopoietic activity of the bone marrow characterized by leucopenia, thrombocytopenia, and hematuria (Tourchi-Roudsari, 2014; Ugochukwu, 2018).

Genotoxic/ Citotoxity activity

Several research works have shown that bracken fern extract induces genetic instability and DNA damage in vitro. However, recent studies revealed that ptaquiloside is directly linked to DNA band breaks in gastric cells in vitro

with decreased cell viability, late apoptotic and necrotic cell induction (Gomes *et al*., 2012). Also, bracken fern induces cell cycle arrest and cellular apoptosis at low and high concentrations respectively (Tourchi *et al*., 2012).

Bracken-fern spores cause the rupture of the DNA strand in human premyeloid leukemia cells *in vitro* (Siman *et al*., 2000). Other studies showed fern extracts induce chromosomal aberrations in mammalian cells (Almeida Santos *et al*., 2006). Ptaquiloside is the main agent of DNA damage induction due to spontaneous deamination and DNA chain breakdown (Gomes *et al*., 2012; Pereira *et al*., 2009). However, Yamada (2007) showed that it was not only this compound that would lead to all these damages, but the other compounds present in the plant**.**

Furthermore, assays with evaluation of p53 gene expression revealed that DNA damage occurs during the first few hours of exposure to gastric epithelial cells (Gomes *et al*., 2012). Mutations in this gene are the most frequent somatic genetic abnormalities detected in human malignant disease, and increasingly tend to be more common in bladder cancer (Hainaut & Hollstein, 1990; Dalbagni *et al*., 2001). The p53 gene is responsible for encoding a transcription factor of 53 kDa, with an important role in DNA repair and cell apoptosis. Thus, the detection of any mutations in p53 genes in fern-induced carcinogenesis will help to better understand the mechanism involved in carcinogenesis (Sharma *et al*., 2013).

Carcinogenic Activity

The first description of the carcinogenic effect of *Pteridium aquilinum* was reported by Rosenbreger and Heeschen (1960), who described hematuria and changes of polypoid nature in the bladder of 5 bovines fed with sublethal fern doses, for long term. Pamucku *et al*. (1976) indicated the relationship between the geographical distribution of ferns and cases of enzootic hematuria. However, the presence of papillomavirus has also involved. Thus, in 1965, fern was reached the carcinogen status by the induction of intestinal adenocarcinomas in rats fed for nine weeks on a diet containing one-third of its weight of dry and ground fern. All animals included in the trial developed neoplasms, often with the formation of diverticula and intussusceptions mainly at the level of the ileum, whereas the animals in the control group did not develop neoplasms lesions (Evans, 1976).

Further research confirmed the carcinogenic activity of the plant, using different laboratory animals, various fern subspecies and different ingestion doses. Laboratory animals, depending on the dose and time of exposure, develop leukemias intestinal neoplasms, pharynx, esophagus, breast

subcutaneous tissue, palate, kidney and bladder (Pamukcu *et al*., 1980; Yoshikawa *et al*., 1981; Hirono, 1989; Shahin *et al*., 1998). Studies on female mice, fed with the fetus and their progeny, revealed maternal adverse effects such as embryo intoxication, alteration in the physical and neurobehavioral development of the offspring, increased incidence of lung tumors and mammary glands tumors (Pamukcu *et al*., 1978; Yoshikawa *et al*., 1981; Hirono, 1989; Salazar *et al*., 1990; Gerenutti *et al*, 1992).

In cattle, bladder tumors have been induced experimentally however, no metastization was observed (Pamukcu *et al*., 1976).

Fresh or dried *Pteridium aquilinum* or its various parts, including spores or extracts can develop tumors lesions in various body locations in different animal species such as cattle, mice, rats, or guinea pigs (Povey *et al*., 1996; Salazar *et al*., 1990).

With the extracts of rhizome, leaves and the whole plant, the mutagenic power of *Pteridium aquilinum* was confirmed in the Ames test, and there was no significant difference in the mutagenicity of extracts from the different parts (Rao *et al*., 1991).

The carcinogenicity effect of bracken-fern was attributed to the chemical compounds ptaquiloside, quercetin and shikimic acid. Toxicity studies of quercetin and shikimic acid indicated that they do not present a genotoxic effect. Quercetin and its derivatives exert cytotoxic effects but are not directly related to the etiological agents of fern intoxication in cattle, since it probably acts as a co-carcinogen agent *in vivo* of bovine papillomavirus (Pamukcu *et al*., 1980; Ngomuo and Jones, 1996).

At some points on the planet, the fern has very high concentrations of ptaquiloside, thus depending on the carcinogenic activity of the plant genotype and the geographical conditions (Smith *et al*., 1992; de Oliveira, 1993).

Regarding ptaquiloside, its concentration in the plant may vary according to the fern variety. Also, the ptaquiloside reactivity is pH-dependent. Thus, at acidic pH, it undergoes aromatization causing pterosin B. The alkaline pH, ptaquiloside is converted to an unstable dienone containing a highly reactive cyclopropyl group that can react rapidly with amino acids, nucleosides, nucleotides, and DNA. The compound causes alkylation of H-ras DNA, primarily in adenine, at codon 61, followed by depuration and error in DNA synthesis, which leads to activation of the H-ras proto-oncogene. The reactions were observed on the N3 and N7 atom of the purines, from the exocyclic atom of guanine and DNA phosphate. The breakages derive from the spontaneous cleavage of the bonds on the N3 adenine and N7 guanine

atoms (Shahin *et al*., 1998; (Prakash *et al*., 1996; Povey *et al*., 1996). In the N3 atom of adenine, the alkylation is preferably in the 5'-TAG and 3'-A sequences at 5'-AA-3', and the N7 in the 5'-TG sequences (Shahin *et al*., 1999; Yamada *et al*., 2007). The formation of bonds between ptaquiloside and DNA is considered the beginning of the tumoral process (Shahin *et al*., 1998).

The ileum and urine of the herbivores have alkaline pH, which may favor the activation of the ptaquiloside (Prakash *et al*., 1996).

Besides, a high frequency of chromosomal aberrations in peripheral lymphocytes was also detected in cattle raised on extensive pasture with fern, probably due to plant carcinogenicity or bovine papillomavirus infection (Moura *et al*., 1988).

Bovine Papillomavirus

The bovine papillomavirus (BPVs) is a double-stranded DNA virus, species-specific that produces cutaneous and mucosal neoplastic lesions (Sharma *et al*., 2013). Bladder cancer in cattle is associated with the presence of papillomavirus in natural and experimental conditions (Campo, 1992; Campo 1997; Cota *et al*., 2015). In cattle, the cutaneous papillomatosis agent has proven to be capable to produce fibroma lesions when injected into the submucosa of the bladder (Olson *et al*., 1959). Although immunosuppression caused by BPV is enough to cause pre-malignant lesions, disease progression occurs only when the mutagenic agents present in bracken fern interact synergically with the BPV causing chromosomal aberrations and clastogenesis. After the development and progression of the neoplasm, BPV-2 expresses the E5 oncoprotein and modifies telomerase activity (Borzacchiello *et al*., 2003). A recent study demonstrated similar synergism between BPV-4 and quercetin, a mutagenic flavonoid presented in bracken fern. This interaction results in gastrointestinal tumors in cattle and humans (Carvalho *et al*, 2006).

Risk Factors

Reports about the risk factors of EBH are scarce. The strongest risk factor for bovine urinary bladder tumors is related to the geographical location where cattle rear in areas with a high density of bracken fern. Thus, cattle only resort to consuming bracken fern when there is little alternative vegetation available, when the fern is still green or when the animals graze in large quantities of this plant (Briggs & Longman 1989; Alonso-Amelot *et al*., 1996: Sharma *et al*., 2013).

The free-grazing of cattle during the spring for two or three months as well as the use of fresh fern as bedding in the spring and summer was also indicated as risk factors (Hidano *et al*., 2017).

Clinical Signs

BHE clinical signs are more evident in females during pregnancy and after parturition. In contrast, the mortality rate is higher in males due to obstruction of the urinary tract by blood clots. However, no sexual predisposition is recognized (Nielson and Moulton, 1990; Sharma *et al*., 2013; Tourchi-Roudsari, 2014). The onset of the disease is presented, on average, at 7-year-old with an expected life spam about 1.5 years (Sharma *et al*., 2013).

Clinical signs may vary according to the severity of hematuria, the stage of disease, and the existence of obstruction and secondary infections. Initially, the irregular presentation of hematuria together with unspecific symptoms such, difficult the diagnosis. Also, the observation of clinical signs is delayed since cattle rear in extensive areas for long periods (Nielsen & Moulton, 1990).

The evolution of BEH is usually slow and intermittent, leading to the progressive emaciation of the animal. The animals usually presents severe hematuria, strangury, anemia with pale mucosa, and absence of fever (Ugochukwu *et al*., 2018). Besides palpation of multiple masses within the bladder wall, cattle also presented weight loss and a decrease in milk yield (Meisner *et al*., 2009).

Ingestion of large amounts of fern during 1-3 months could lead to an acute toxicosis with as fever, lethargy, drooling, hemorrhages of varying degrees in several organs like the gums, nostrils, and gastrointestinal tract, pancytopenia, hematuria, bone marrow suppression or blood in the milk may occur. (Anjos *et al*., 2008; Ugochukwu, 2018)

Clinical Pathology

Regarding laboratory analysis, blood tests displayed non-regenerative anemia usually associated with thrombocytopenia and leucopenia (Rosemberger *et al*., 1983; Giles & Andrews, 2008).

Urine analysis is needed to the verification of hematuria. However, this clinical sign is unspecific since it can be present in other clinical conditions. Urine microscopic analysis should be considered a reference diagnostic test. At the farm, diagnostics of hematuria are usually carried out by the use of commercial urine dipsticks (Pavelski *et al*., 2014). Microscopic analysis of urine increases the diagnostics of BEH since hematuria can be quantified by erythrocyte count. Also, examination of urinary sediments exhibited the presence of phosphate crystals and neutrophils. Bacteriological examination of urine samples does not reveal any significant result (Dawra *et al*., 1991).

The hematological profile is characterized by progressive anemia and leucopenia (Prasad, 1983). Antithrombin III, protein S activities, protein C, and fibrinogen plasma levels are usually increased (Di loria *et al*., 2012).

Biochemical analysis showed an increase in blood urea nitrogen and serum creatinine levels indicating some involvement of the kidney in the pathogenesis of EBH (Prasad & Iyer, 1986). Therefore, increased levels of the protein and creatinine concentrations in the urine of cattle affected by EBH have been also referred (Dawra, *et al*., 1991).

Necropsy and Histopathological Findings

HEB lesions are observed in the bladder and occasionally in renal pelvis or ureters. They include petechiae, ecchymosis or hemorrhagic suffusions, discoloration, and neoplasias (figure 2).

Tumors often occur in the ventral and lateral wall of the *fundus* or the trine. They presented as polyps, single or multiple, and often are pedunculated or sessile. Some contain mucin cysts (Nielsen & Moulton, 1990).

Local invasion and metastasis in the iliac lymph nodes or lungs may be observed (Maxie, 1993; Carvalho *et al*., 2006; Cota *et al*., 2015).

Other lesions, such as infarcts in the liver, bone marrow aplasia and tumors in pharynx, esophagus, and rumen might also be found (Anjos *et al*., 2008; Ugochukwu, 2018).

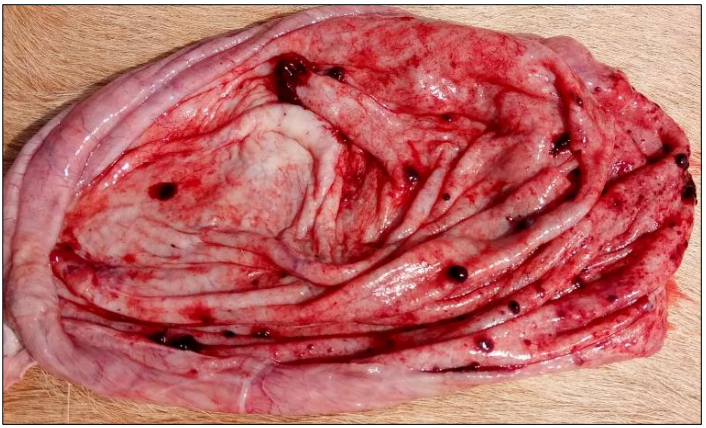

Fig 2: BEH - bladder tumours (courtesy of Dr. Carlos Jardim de Freitas and Dr. Daniel Bravo da Mata)

EBH is associated with epithelial and connective bladder tumors, including papillomas, transitional carcinomas, adenomas, fibromas, fibrosarcomas, leiomyosarcomas, hemangiomas, and angiosarcomas. Multiple tumors of one or more types may be present in the same animal, being the most frequent association of papillomas, fibromas, and hemangiomas with

carcinomas. Other histological alterations that may be observed are focal hyperemia, dilation of the blood vessels of the mucosa and submucosa, edema, petechial hemorrhages, ulcers, and chronic cystitis. Urothelium exhibits metaplastic changes (squamous or mucosal metaplasia) or hyperplastic, with the appearance of von Brünn nests (Carvalho *et al*., 2006; Pires *et al*., 2009; Pires *et al*., 2010).

Diagnosis

The diagnostics of EB should be made by a combination of the anamnesis (access to fern), clinical signs, and different complementary diagnostics tests. Besides blood and urine analysis, ultrasonography has been described for BEH to observe alterations in urine bladder. Since, EBH has been related to bovine papillomavirus, its detection by PCR may also improve the diagnostics. *Post mortem* examination and histopathology allow the definitive diagnosis (Prasad, 1983; Hoque *et al*., 2002).

Public Health

Bracken fern has long been used by man by for animal bedding, medicinal properties, or as fertilizer, among others (Lee & Shin, 2011). However, the potential threat of bracken fern for public health has increased in the last years due to the presence of carcinogenic components in its constitution such as ptaquiloside (dos Reis Aranha, *et al*., 2019).

Human contamination may be direct from fern exposition or indirect, by the ingestion of cattle products. Direct exposition to bracken carcinogens include inhalation of bracken spores (Povey *et al*., 1996; Rasmussen *et al*., 2013) or ingestion of contaminated water. A study by Siman *et al*. (2000) showed that several spore extracts from different ferns, including *Pteridium aquilinum*, cause DNA damage. However, the pathological mechanism of tumor development is still unknown. Ptaquiloside leaching of the fronds exposed to rain may subsequently contaminate soil and groundwater (Jatoba *et al*., 2016). It is also known that some of the components present in the fern are soluble and hence water is a possible source of contamination although with a scarce risk compared to milk or meat ingestion (O´Driscoll *et al*., 2016).

Ingestion of the fern as food has been associated with high levels of esophageal cancer in Japan and esophageal and gastric cancer in Brazil (Povey *et al*., 1996). In Japan, young leaves are usually eaten as an appetizer, raw or cooked. From the dried and peeled rhizomes, flour is made with the so-called bread of the fern, used by the poorest people or in times of famine. References to this type of bread are found in France, England, New Zealand, and Santa Maria Island. The American Indians eat the rhizome (Fernandes & Fernandes, 1983; Rymer, 1976; Santos, 1990).

Indirect exposure of man is related to milk and meat of animals with EBH. Carcinogenic compounds present in fern can be present from milk and meat as chemical residues. The carcinogenic effect of ptaquiloside by milk ingestion is still unknown although some studies related a high incidence of gastric tumors in farmers' population of Costa Rica, Central America and Southern Mexico that consume milk from cattle raised in high bracken fern density area (Alonso-Amelot *et al*., 1996; Povey *et al*., 1996). Also, it has been observed that milk from cows that consumed fern displayed a carcinogenic effect in rodents (Pamukcu *et al*., 1980). Rats fed with fresh and/or powdered

milk showed a higher incidence of bladder carcinoma, suggesting the presence of carcinogenic metabolites in milk (Vetter *et al*., 2018). Ptaquiloside content has been quantified in milk from cows fed with *P. aquilinum* var. *caudatum.* Thus, these results may have implications in newborns and infants fed with cow's milk from pastures with a high density of bracken ferns (Salazar *et al*., 1990). Thermal processing of milk seems to inactivate ptaquiloside (Villalobos-Salazar *et al*., 2000). Therefore, a potential public health concern for urban populations is scarce (Alonso-Amelot *et al*., 1998). However, the rise of green consumerism, including the current tendency to drink raw milk, may have a negative impact on public health with special importance of those milk from cattle raised on high-density bracken fern areas.

Regarding the presence of ptaquiloside and related terpene glycosides in meat, its potential impact as chemical hazards has been discussed (Gil da Costa *et al*., 2012). Thence, a report by the British Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) has recently addressed the risks placed by ptaquiloside-contaminated foodstuffs and considered it reasonable to regard bracken as potentially carcinogenic at all levels of ingestion. Committee on toxicity of chemicals in food, consumer products, and the environment also called for further studies on ptaquiloside residues present in foods derived from bracken-fed animals and on the determination of safety intervals for ptaquiloside clearance from tissues and milk (COT, 2008). Thus, the presence of ptaquiloside is the skeletal muscles and liver have reported in bracken-fed cattle (Fletcher *et al*., 2011).

Conclusion

Bovine enzootic hematuria is a syndrome, considered endemic in certain areas of the planet. It is characterized by hematuria, associated in most cases with bladder neoplasms. Although viral agents may be involved, the etiology of this process is mainly a management problem, associated with the conditions and type of feeding of the animals. The fern *Pteridium aquilinum* is responsible for the contribution of carcinogens, and its ingestion is associated with neoplasms in humans and animals. This issue has aroused our interest, both by the worldwide abundance of the fetus and by its repercussions on public health.

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