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Clotting profile in cattle showing chronic enzootic haematuria (CEH) and bladder neoplasms

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ABSTRACT

Primary haemostasis (bleeding and blood clotting time), activated partial thromboplastin time (APTT), prothrombin time (PT), antithrombin III (ATIII), protein C, protein S, fibrinogen and D-dimer were determined in 13 cattle affected by chronic enzootic haematuria (CEH) and bladder neoplasms and 10 healthy cattle (control group). Increases in antithrombin III and protein S activities ($P < 0.01$) and protein C and fibrinogen plasma levels ($P < 0.05$) were observed in sick animals, while activated partial thromboplastin time, prothrombin time, and D-dimer did not show significant differences when compared to healthy animals. The clotting profile observed does not seem responsible for the chronic bleeding typical of CEH. The observed modification of some coagulation markers may derive from multiple interactions among cancer, inflammation and viral infection status typical of this syndrome.

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1. Introduction

Chronic enzootic haematuria (CEH) is a syndrome commonly associated with tumours of the bladder in cattle. Different factors, such as chronic ingestion of bracken fern (*Pteridium aquilinum*) and bovine papilloma virus type 2 (BPV-2) infections, seem to be involved in the aetiopathogenesis of the syndrome. The progression of urinary bladder lesions to neoplasia depends on an interrelationship between papilloma virus infection and carcinogenic, mutagenic, clastogenic and immunosuppressive compounds of bracken fern such as quercetin and ptaquiloside (Reddy and Fialkow, 1983; Campo et al., 1992; Campo, 1997).

P. aquilinum is commonly present in pastures of southern Italy, and the prevalence of CEH associated with bovine bladder neoplasia is high (Borzacchiello et al., 2003). Clinically, CEH is characterised by anaemia, haematuria, progressive emaciation, and finally death (Hopkins, 1986). Furthermore, severe non-regenerative anaemia, thrombocytopaenia, leukopaenia with lymphopaenia and neutropaenia are commonly observed in affected animals (Maxie and Newman, 2007; Marrero et al., 2001; Van Metre and Divers, 2002; Divers, 2007). The haematuria observed in CEH is the result of vascular changes due to haemorrhagic cystitis and/or urinary bladder tumours (Maxie and Newman, 2007). Histopathologically, the neoplasms are of urothelial, mesenchymal (vascular), and mixed urothelial–mesenchymal types (Eble et al., 2004; Roperto et al., 2010). The presumed diagnosis of CEH is based on the presence of micro–macrohaematuria, weight loss, anaemia and known access of cattle to bracken fern. However, post-mortem examination is necessary to confirm the origin of the haematuria although cystoscopy and/or ultrasound examination have recently been proposed as useful tools for an ante-mortem diagnosis (Franz et al., 2004).

Several studies in human and veterinary medicine have shown that urinary tumour cells possess the ability to interact with all the components of the haemostatic system, activating both the coagulation cascade and stimulating the prothrombotic properties or fibrinolysis; the activation of the coagulation system can then facilitate the tumour growth, neo-angiogenesis and metastasis (O'Keefe and Couto, 1988; Rickles et al., 1992). In particular, human patients with urinary bladder carcinoma showed an activation of the coagulation cascade with an increase in fibrinolysis and antithrombin III activity (Iwan-Zietek et al., 1997; Zietek et al., 1997). In veterinary medicine, the knowledge of clotting profile in cattle with CEH is poor and fragmentary. In a recent paper, Perez-Alenza et al. (2006) reported only thrombocytopaenia in almost all animals affected by CEH.

The aim of this study was to evaluate the clotting profile (primary haemostasis tests, APTT, PT, fibrinogen, D-dimer, ATIII, protein C and protein S) of cattle with CEH and bladder neoplasms, in order to

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understand the pathogenesis of chronic bleeding observed in this syndrome.

2. Materials and methods

2.1. Selection of animals

One hundred Podolica cattle breed aged 3–20 years were enroled from farms located in southern Italy, where *P. aquilinum* is commonly present. After a complete physical examination with particular attention for the body condition score, urine samples were collected from each animal, either by free-catch technique or urethral catheter. The samples were tested using chemical multiple-reagent commercial strips (Combur test Roche – Swiss) in order to detect the presence of micro/macro haematuria. In animals showing haematuria, microscopic examination of the urine sediment after centrifugation was carried out to confirm the presence of red blood cells and to rule out the presence of parasites (e.g. *Shistosoma bovis*). Fifteen animals with haematuria were selected.

2.2. Cystoscopy procedure

Cystoscopic examination was performed in order to evaluate the appearance of the urinary bladder mucosa. Each cow was restrained in a stanchion and received a local regional anesthetic (lidocaine 2%, Fort Dodge Animal Health, Italy). A bovine vaginal speculum was used to identify the urethra. Multiple cystoscopic investigations were carried out using a 30° rigid endoscope (Karl Storz – Germany), 40 cm in length and 4 mm in diameter, slowly inserted in the urethra. In order to obtain an accurate view of the urinary mucosa, the urinary bladder was irrigated with 1 L of sterile 0.9% NaCl. A xenon light source (300 w 6000 K – Karl Storz – Germany) and a camera (Mono CCD25 mm – Karl Storz – Germany) connected to the cystoscope allowed visualisation and recording of all diagnostic images. The status of the urinary bladder mucosa was assessed and colour, smoothness, brightness, vascularization and pathological findings recorded.

2.3. Histopathological and molecular examination

The cystoscope shaft 28 Ch included a working channel through which biopsy forceps (fenestrated grasping and Blakesley type) were introduced. During the examination, multiple biopsies of the urinary bladder mucosa were taken and placed in 10% buffered formalin; some were embedded in paraffin, cut at 4 μm and stained with haematoxylin and eosin for histopathological examination. Neoplasms were classified according to the criteria recently reported (Roperto et al., 2010).

Molecular procedures were carried out to detect bovine papilloma virus type 2. In particular, western blot examination was performed to show the expression of E5 protein, known to be the major oncoprotein of BPV-2 as elsewhere reported (Roperto et al., 2008).

2.4. Coagulation and haemato-biochemical analyses

Coagulation and haemato-biochemical profiles were obtained in all affected animals as well as in 10 healthy cows (control group). A complete clinical examination, with special attention to the urinary tract, along with complete blood and urine analyses, were carried out in all animals. Blood samples were collected from the caudal tail vein and transferred to plastic tubes containing EDTA, sodium citrate 3.8% and serum separator. Haemocytometric analyses were performed within 1 h of collection using a laser cell blood counter (ADVIA 120, Bayer, Germany). Red cells (RBC), haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white cells (WBC), leukogram and platelets (PLT) were analysed. The differential leucocyte count (a minimum of 200 white cells) was performed using May-Grunwald-Giemsa stained blood smears; at the same time, routine review of the peripheral blood cells was carried out to assess platelets, red blood cell morphology and the presence of blood parasites.

After centrifugation, serum was also collected, stored at −20 °C and thawed at 37 °C, immediately before use; serum urea, creatinine, glucose, creatinine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), total bilirubin, cholesterol, triglycerides and serum protein electrophoresis were analysed using commercial kits (Reactivos Spinreact S.A. OLOT, Gerona, Spain).

Finally, in the animals with haematuria, consequent to urinary neoplasms, primary and secondary haemostasis were also evaluated. After primary haemostasis, tests such as bleeding and blood clotting times were carried out whereas secondary hemostasis was tested evaluating activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen, D-dimer (immunological method), antithrombin III (ATIII), protein C, (chromogenic method), and protein S (immunoturbidimetric method) via an automated coagulometer (ACL 9000) using the commercial kit HemosILTM (Instrumentation Laboratory Company – USA). The measurements obtained from plasma samples were extrapolated on a calibration curve obtained from scalar dilutions of calibration plasma. Inter-assay and intra-assay coefficients of variation for ATIII, Protein C, and Protein S determinations were <5%, <8%, <7%, respectively.

2.5. Statistical analysis

Data were analysed by the student *t*-test for significance. *P* value < 0.05 was taken as the level of significance.

3. Results

3.1. Physical examination

The clinical history revealed intermittent haematuria for at least 2 years prior to the visit. A body condition score between 1 and 2 was shown in 87% (13/15) of the cattle. Mild dehydration (5%) was present in all animals, while ocular mucosa was pale in nine animals (60%). A slight increase in capillary refill time (5 s) was observed in seven animals (47%). Tachypnoea (30–35/min) and high pulse rate (78–90/min) were present in all affected animals. However, none had abnormal rectal temperature or lymph nodes. Twelve animals (80%) showed severe macrohaematuria, with two cows affected by dysuria/stranguria. Rectal palpation showed a thickening of the bladder wall associated with pain reaction in five of 15 animals.

3.2. Urine analysis

Twelve affected animals showed gross haematuria with opaque-turbid, red-brownish coloured urine with a haematic odour; only three animals showed microhaematuria. The urine specific gravity showed a mean value of 1024 ± 7. The pH was alkaline with values between 8 and 9. Microscopic examination of urine sediment confirmed the presence of intact red blood cells, leucocytes and epithelial cells.

3.3. Cystoscopy

Cystoscopy revealed large proliferative masses (vegetated) on the vault and lateral wall of the bladder in three animals; nine

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