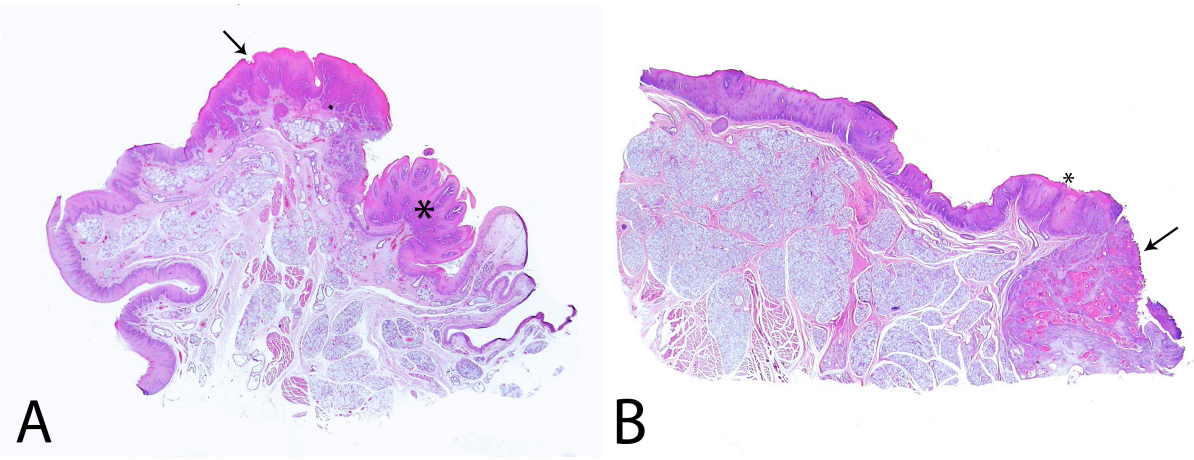

Fig. 4. Squamous cell carcinomas (SCC). (A) Invasive SCC in initial stage of growth (arrow) and a developing papilloma (asterisk). There is no morphologic evidence of a papillomatous transformation to carcinoma in this area. Haematoxylin and eosin. Objective, 2.5x. (B) Invasive SCC in advanced stage of growth (arrow). A severelly dysplastic epithelium (asterisk) seems to give rise to the well-developed invasive SCC. Haematoxylin and eosin. Objective, 2.5x.

4. ARTIGO 2

SQUAMOUS CELL CARCINOMA OF MINOR SALIVARY GLANDS IN CATTLE GRAZING ON BRACKEN FERN: IMPLICATIONS ON PATHOGENESIS

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Squamous cell carcinoma of minor salivary glands in cattle grazing on bracken fern: implications on pathogenesis

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Abstract

Squamous cell carcinomas (SCCs) of the upper digestive tract (UDT) occur in cattle grazing on bracken fern (*Pteridium aquilinum*). The pathogenesis is related with the development of papillomas and a possible further transformation to carcinomas. However, morphological evidences of SCCs possibly arising from minor salivary glands may represent an alternate pathogenetic pathway of the disease. The aim of this study was to evaluate a possible minor salivary gland origin for alimentary SCCs of cattle grazing on bracken fern. Forty SCCs of the UDT were evaluated. Nine SCCs seemed to be arising from minor salivary glands and had pools of Alcian blue-positive mucus inside island of neoplastic keratinocytes. A strong immunohistochemical evidence of a SCC arising from a minor salivary gland positively stained for cytokeratin 7/8 was observed. This study provides morphologic evidences that SCCs arise in minor salivary glands and it may result from a direct chemical carcinogenic effect.

Keywords: cattle, bracken fern, *Pteridium aquilinum*, squamous cell carcinoma.

Introduction

Bracken fern (*Pteridium aquilinum)* is the only known plant that naturally causes cancer in animals (Yamada *et al.* 2007). Epidemiologic studies suggest that the plant may be also involved in some cases of human gastric cancer (Alonso-Amelot and Avendano 2001, Alonso-Amelot and Avendano 2002).

In cattle, chronic poisoning by bracken fern is associated with two types of clinical disease: urinary bladder tumours (bovine enzootic hematuria - BEH) (Price and Pamukcu 1968, Campo *et al.* 1992, Gabriel *et al.* 2009) and squamous cell carcinoma (SCCs) of the upper digestive tract (UDT) (Campo *et al.* 1994, Souto *et al.* 2006, Masuda *et al.* 2010). In

both cases, grazing for long periods of time on pastures highly contaminated with bracken fern is the major factor associated with these diseases, since they do not occur in areas without the plant (Döbereiner *et al.* 1967, Jarrett 1978).

The norsesquiterpene ptaquiloside is the major chemical compound of this fern that has been proved to be radiomimetic, mutagenic and carcinogenic (Fenwick 1989). Ptaquiloside bounds to DNA forming adducts, the basis for chemical carcinogenesis (Yamada *et al.* 2007). The ptaquiloside can also be transformed in alkaline pH solutions, producing a highly mutagenic dienone (Fenwick 1989, Yamada *et al.* 2007). The activation to dienone is an important feature in the pathogenesis of chronic bracken fern poisoning and it is closely related to the location of tumours since saliva in the UDT and urine in the urinary bladder have alkaline pH in normal conditions, 7.8 and 8.5, respectively (Fenwick 1989).

High doses of ptaquiloside exert a potent radiomimetic effect in animals (Hirono *et al.* 1984). High intake of bracken fern in a short period of time leads to the development of severe bone marrow aplasia as part of the acute hemorrhagic syndrome in cattle (Evans 1968, Hirono *et al.* 1984, Anjos *et al.* 2009).

Quercetin, a flavonoid, is also implied as an important mutagenic chemical compound of bracken fern (Bjeldanes and Chang 1977, Pamukcu *et al.* 1980). Several studies *in vitro* have demonstrated that quercetin acts in synergism with bovine papillomavirus type 4 (BPV-4) promoting malignant transformation of primary bovine cells (Pennie and Campo 1992, Cairney and Campo 1995, Beniston *et al.* 2001).

Papillomavirus is a double-stranded DNA virus of the family Papillomaviridae (Howley and Lowy 2006). The development of papillomas, a benign epithelial tumour, is dependent on epithelial stratification, expressing different viral open reading frames (ORFs) in different layers of the squamous epithelium (Zur Hausen 2002). Thus, productive papillomaviral infection only occurs in keratinocytes undergoing squamous terminal differentiation (Jarrett 1985, Howley and Lowy 2006). In BPV-4 infection, viral particles are mostly found in developing papillomas of the UDT (Jarrett 1980, Campo *et al.* 1985). Small amounts of BPV-4 DNA have been identified in alimentary SCCs of cattle grazing on bracken fern, suggesting its role in the pathogenesis of the disease (Campo *et al.* 1994).

Alimentary papillomatosis is a common finding in cattle grazing for long periods of time in bracken fern (Döbereiner *et al.* 1967, Campo *et al.* 1980, Jarrett 1980). Some authors suggest that the requirement for the formation and maintenance of these papillomas is a chronic and persistent immunosupression state induced by the bracken fern toxic principles (Jarrett 1985, Campo and Jarrett 1986, Campo *et al.* 1994). In the pathogenesis of alimentary

SCCs of cattle grazing on bracken fern, it was hypothesized that these persistent papillomas would act as a large expanded target for bracken carcinogens (Campo *et al.* 1994, Campo 1997). According to this theory, the transformation from papilloma to carcinoma, named papilloma-carcinoma syndrome, would be the pathogenetic basis of these SCCs (Campo and Jarrett 1986, Campo *et al.* 1994).

However, a recent study showed morphological evidences of SCCs possibly arising from minor salivary glands of cattle grazing on pastures contaminated with bracken fern (Masuda *et al.* 2010). This intriguing observation may represent an alternate pathogenetic pathway of the disease. The aim of this study was to evaluate a possible minor salivary gland origin for SCCs of cattle chronically and spontaneously poisoned by bracken fern.

Materials and methods

Forty SCCs were evaluated from cattle necropsied between years 2003 and 2009, which have been grazing on highly contaminated areas with bracken fern. Thirty SCCs were located in the cranial region of the UDT, including the base of tongue, oropharynx and epiglottis. The other ten SCC were located in the lower part of the oesophagus and in the entrance of the rumen. Oesophageal and rumenal SCCs were utilized as negative controls for the presence of minor salivary glands. Clinical and necropsy data were recorded for all cases.

The SCCs were fixed in 10% buffered formalin, routinely processed for histopathology and stained with hematoxylin and eosin. Salivary mucopolysaccharides were demonstrated through histochemical staining with 1% Alcian blue, diluted in 3% acetic acid solution, for 20 minutes. The tumours were histopathologically graded as well, moderate or poorly differentiated SCCs (Head *et al.* 2002, Masuda *et al.* 2010).

All forty SCCs were submitted to the immunohistochemistry technique using the streptavidin-biotin-peroxidase method. Briefly, sections (3μm) were dewaxed, rehydrated through xylene and graded alcohols and rinsed in tap water. Sections were microwaved for antigen retrieval in TRIS-EDTA (pH 9.0) for 10 min. Epithelial cells in general were immunolabelled with rabbit polyclonal anti-bovine pan-cytokeratin (Dako Corp., Carpinteria, California; 1 in 2,000 dilution) and epithelial glandular single-layered cells were labeled with monoclonal anti-human cytokeratin 7/8 (clone CAM5.2; Becton-Dickinson, San Jose, California, ready to use). Primary antibody was incubated for two (pan-cytokeratin) or three hours (for cytokeratin 7/8) at 37^{o}C in a humid chamber. Incubations with secondary

biotinylated antibody and enzyme complex (LSAB-HRP Kit; Dako Corp., Carpinteria, California) were for 35 min each at room temperature and the chromogen-substrate reaction was with 3,3‘diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, Missouri). The sections were counterstained with Harris hematoxylin and Alcian blue. Previously known SCCs of cattle and bovine epididymis were used as positive control for pan-cytokeratin and cytokeratin 7/8, respectively. As negative control, the primary antibody was replaced by phosphate-buffered saline (PBS) during the incubation.

Results

All animals presented progressive weight loss, dysphagia, sialorrhea or ruminal atonia. At necropsy, there were mild to severe papillomatosis, small mucosal nodules, erosions/ulcers and one large SCC, ranging from 8 to 35cm in diameter. Metastases to regional lymph nodes were observed in 50% of the cases.

In the SCCs of the cranial region of the UDT, 8 were located in the base of tongue, 20 in the oropharynx and 2 in the epiglottis (Fig. 1A-C). Histopathological, histochemical and immunohistochemical results are presented on Table 1. The tumours were mostly composed of islands, strands and cords of neoplastic keratinocytes with dyskeratosis and keratin pearls. Oesophageal and ruminal SCCs were grossly and microscopically similar to the tumours of the cranial region. Histopathological grading of all SCCs revealed 27 cases of well-differentiated carcinomas, 8 cases of moderate, and 4 cases of poorly differentiated SCCs. In the SCCs of the cranial region, minor salivary glands were surrounded by groups of neoplastic keratinocytes and accompanied by mild to severe desmoplastic and/or inflammatory reaction. Minor salivary glands were not present in oesophageal and rumenal SCCs. Nine SCCs of the base of tongue, oropharynx and epiglottis seemed to be arising from minor salivary glands.

Acini and ducts of normal minor salivary glands were filled with a strongly positive amorphous Alcian blue-stained mucus in all SCCs of the cranial region of the UDT. None oesophageal or rumenal SCC had any Alcian blue-stained material. The nine cases of suspected salivary gland origin had pools of amorphous Alcian blue-positive material inside island of neoplastic keratinocytes (Fig. 2). Neoplastic cells were embedded in a bluish mucus, mixed in areas of lamellar keratinization.

All SCCs stained for pan-cytokeratin (wide spectrum screening) in immunohistochemistry. The immunolabelling was cytoplasmic, fibrilar and diffuse in neoplastic keratinocytes and finely granular in salivary acini and ducts. The Figure 3 shows positivity for pan-cytokeratin in a SCC of minor salivary gland.

Strong positive staining for cytokeratin 7/8 was observed in acini and ducts of normal minor salivary glands in all SCCs of the cranial region. No immunostaining was observed in normal squamous stratified epithelium or in the oesophageal and rumenal SCCs. Neoplastic keratinocytes were negatively stained for cytokeratin 7/8 in all SCCs of this study. Nevertheless, four pharyngeal SCCs had strongly-stained individual cells inside islands of neoplastic keratinocytes (Fig 4). There were no differences in the staining pattern between the three histopathological stages. A strong immunohistochemical evidence of a SCC arising from a minor salivary gland positive-stained for cytokeratin 7/8 was observed in one case (Fig. 5).

Discussion

In the present study, SCCs of the cranial region of the UDT were in close association with minor salivary glands. However, SCCs around salivary acini does not mean a glandular origin. Areas with glands may have been infiltrated by adjacent neoplastic keratinocytes (Head *et al.* 2002). The differentiation between infiltration of superficial mucosa SCCs and SCCs of minor salivary glands is quite difficult and sometimes considered unreliable (Lewis and Olsen 2005). Nevertheless, there are some unequivocal cases of SCCs of minor salivary glands in the literature (Vered *et al.* 2003, Akrish *et al.* 2009, Brennand-Roper *et al.* 2010). In the present study, there were nine suspect cases of minor salivary gland origin and a confirming diagnosis of SCC was made based on the presence of Alcian blue-positive mucus inside islands of neoplastic cells and on immunohistochemical results.

Immunolabelling for cytokeratin 7/8 is specific for simple glandular epithelia and it does not stain squamous epithelium (Dabbs 2006). The morphological and immunohistochemical transition from a cytokeratin 7/8-positive glandular epithelium to a negative-stained SCC was confirmative of a minor salivary gland origin. Also, pools of alcian-blue positive salivary mucus were observed inside neoplastic islands.

Staining for cytokeratin 7/8 in individual cells inside neoplastic islands in four SCCs was considered an intriguing finding. It may represent a vestige of the glandular origin in the

SCCs of this study. Considering the specific staining for intermediate filaments, such as cytokeratins, these immunostained cells could also reflect distinct cellular properties and different stages of epithelial differentiation (Dabbs 2006, Fillies *et al.* 2006). In general, cytokeratin expression patterns are highly conserved and an alteration in the expression of these specific intermediate filaments is related to substantial changes in the cell behaviour (Hendrix *et al.* 1996, Fillies *et al.* 2006). Recent cell culture and studies with chemical carcinogens have revealed an important role of cytokeratin 7/8 in various steps of the pathogenesis and progression of SCCs (Fillies *et al.* 2006). Transfection of buccal mucosal cells with a vector for cytokeratins 7/8 resulted additionally in significant alterations in the cell morphology and motility, prerequisite features for invasive tumour behaviour (Chu *et al.* 1996, Hansson *et al.* 2003, Raul *et al.* 2004). Nevertheless, expression of cytokeratins 7/8 in individual cells inside neoplastic islands was not related with the histopathological grade of the SCCs of this study.

Although there is an undoubtiful morphological evidence of transformation from a simple glandular to a neoplastic squamous stratified epithelium, non-neoplastic reactive ductal change and other neoplasms were included in the differential diagnosis. Patchy hyperplasia and squamous metaplasia of interlobular ducts may be induced by vitamin A deficiency and highly chlorinated naphtalene poisoning (Head *et al.* 2002, Brown *et al.* 2007). Similar changes are seen in ducts at the periphery of necrotic tissue in salivary infarction in dogs, cats and humans (Kelly *et al.* 1979, Eisenberg 1986, Brown *et al.* 2007). However, there was no clinical or pathological evidence of vitamin A deficiency or salivary gland infarction in any animal of this study. Mucoepidermoid carcinomas (mixed epidermoid or mucus-secreting carcinomas) differ from salivary gland SCCs because they are composed of a mixture of neoplastic keratinocytes, mucous cells and intermediated type cells (Koestner and Buerger 1965, Karbe and Schiefer 1967, Ellis and Simpson 2005). Despite the abundant amount of salivary mucus inside island of neoplastic keratinocytes, mucous cells and intermediated type cells were not present, favoring the diagnosis of SCCs.

Minor salivary gland SCCs in cattle grazing on bracken fern may represent an important finding for the etiopathogenesis of the disease. The hypothesis of the papilloma-carcinoma syndrome was established after an experimental study with cattle published in 1994 (Campo *et al.* 1994). It was hypothesized that the development and persistence of the papilloma was a crucial step for carcinomatous transformation to SCCs (Campo *et al.* 1994, Campo 1997). However, based on the results of this study, the sequential progression from

papillomas to carcinoma may not be the only pathway to the development of SCCs of the UDT in cattle grazing on bracken fern.

In the present study, neoplastic transformation of minor salivary glands could not be associated with a papillomatous formation, but it could be closely related to the direct carcinogenic effect of ptaquiloside. This hypothesis is based on the single cell layer epithelium of salivary glands, which expresses cytokeratin 7/8 (Dabbs 2006). The complete formation of a papilloma, for further transformation to SCC, would not be possible without the presence of epithelial stratification, as it occurs in the squamous stratified epithelium of the UDT. Therefore, the papilloma would not be able to grow and to act as an expanded target for bracken carcinogens. However, abortive replications of BPV-4 (or other BPV) (Borzacchiello *et al.* 2003) cannot be completely ruled out.

Ptaquiloside can induce tumours in several animal species (Evans and Mason 1965, Evans 1968, Pamukcu *et al.* 1972, Hirono *et al.* 1987, Shahin *et al.* 1998). A potent carcinogen-mutagen dienone is formed when ptaquiloside is submitted to alkaline solutions (Fenwick 1989, Vetter 2009). In cattle grazing on bracken, such chemical metabolism has a strong correspondence to the location of tumours. Syndromes like SCCs of the UDT and tumours in urinary bladder in BEH of cattle are closely related to the alkaline pH of the saliva and urine, respectively (Fenwick 1989). The multiplicities of tumours in different epithelia are indicators of the degree of carcinogen exposure or general organism-wide susceptibility to carcinogenesis. Multiple epithelia are affected when a chemical compound is systemically metabolized (Ha and Califano 2003).

Ethanol-induced cancers in humans have some similarities to bracken fern-related tumours of cattle. In human ethanol-related head and neck SCCs, saliva is also an important factor involved in carcinogenesis (Simanowski *et al.* 1993). Acetaldehyde, a carcinogen to several animal species and formed when ethanol is metabolized by oral bacteria, is the most important cancer-causing agent in the UDT of humans (Seitz *et al.* 1990, Homann *et al.* 1997). The chemical basis for carcinogenesis of acetaldehyde is the formation of DNA adducts (Seitz and Stickel 2007). Acetaldehyde concentration in saliva are 10-100 times higher than in blood (Homann *et al.* 1997). As consequence of a high acetaldehyde concentration in a hyper-regenerative environment, the generation of highly mutagenic acetaldehyde-DNA-adducts is facilitated in these tissues (Simanowski *et al.* 1993, Seitz and Stickel 2007). Similarly in cattle grazing on bracken fern, high concentrations of ptaquiloside-DNA-adducts and dienone in salivary glands and saliva could be the responsible for induction of SCCs of the UDT. The induction of these changes could be the result of exposure of the

ducts to either penetration of the carcinogen through the oral mucosa or retrograde passage of the carcinogen from the oral cavity through the duct lumen (Vered *et al.* 2003). In the present study, some carcinomas may have been arisen from direct carcinomatous transformation of minor salivary glands.

In conclusion, this study provides morphologic evidences of SCCs arising in minor salivary glands in cattle grazing for long periods on bracken fern. This finding adds relevant data for further studies on pathogenesis of alimentary SCCs of cattle.

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Table 1 – Morphological, histochemical and immunohistochemical results of bovine alimentary SCCs.

Case	Location	Histological stage †	Alcian blue †	IHC Pan-Cytokeratin †	IHC Cytokeratin 7/8 †	
					Normal minor salivary gland	SCC
1	Base of tongue	WD	+	+	+	-
2	Oropharynx	MD	+	+	+	$+^{\S}$*
3	Epiglottis	WD	-	+	+	-
4	Oropharynx	MD	-	+	+	+*
5	Base of tongue	WD	-	+	+	-
6	Base of tongue	WD	-	+	+	-
7	Base of tongue	WD	+	+	+	-
8	Oropharynx	WD	-	+	+	-
9	Base of tongue	WD	-	+	+	-
10	Oropharynx	WD	+	+	+	+*
11	Oropharynx	WD	-	+	+	-
12	Oropharynx	WD	+	+	+	-
13	Oropharynx	WD	-	+	+	-
14	Oropharynx	WD	+	+	+	-
15	Oropharynx	WD	-	+	+	-
16	Oropharynx	WD	-	+	+	-
17	Oropharynx	WD	-	+	+	-
18	Oropharynx	MD	-	+	+	-
19	Epiglottis	MD	+	+	+	-
20	Oropharynx	WD	-	+	+	-
21	Oropharynx	MD	+	+	+	-
22	Base of tongue	WD	-	+	+	-
23	Oropharynx	WD	+	+	+	+*
24	Base of tongue	PD	-	+	+	-
25	Oropharynx	WD	-	+	+	-
26	Oropharynx	WD	-	+	+	-
27	Base of tongue	WD	-	+	+	-
28	Oropharynx	MD	-	+	+	-
29	Oropharynx	WD	-	+	+	-
30	Oropharynx	MD	-	+	+	-
31	Rumen	PD	-	+	n/a	-
32	Rumen	PD	-	+	n/a	-
33	Rumen	WD	-	+	n/a	-
34	Rumen	MD	-	+	n/a	-
35	Rumen	WD	-	+	n/a	-
36	Oesophagus	WD	-	+	n/a	-
37	Oesophagus	PD	-	+	n/a	-
38	Oesophagus	WD	-	+	n/a	-
39	oesophagus	WD	-	+	n/a	-
40	Oesophagus	MD	-	+	n/a	-

† WD: well-differentiated; MD: moderated differentiated; PD: poorly differentiated; +: positive; -: negative; n/a: not available; §: transition between positive cytokeratin 7/8 minor salivary gland to negatively-stained SCC; *: immunolabelling in individual cells inside neoplastic islands.

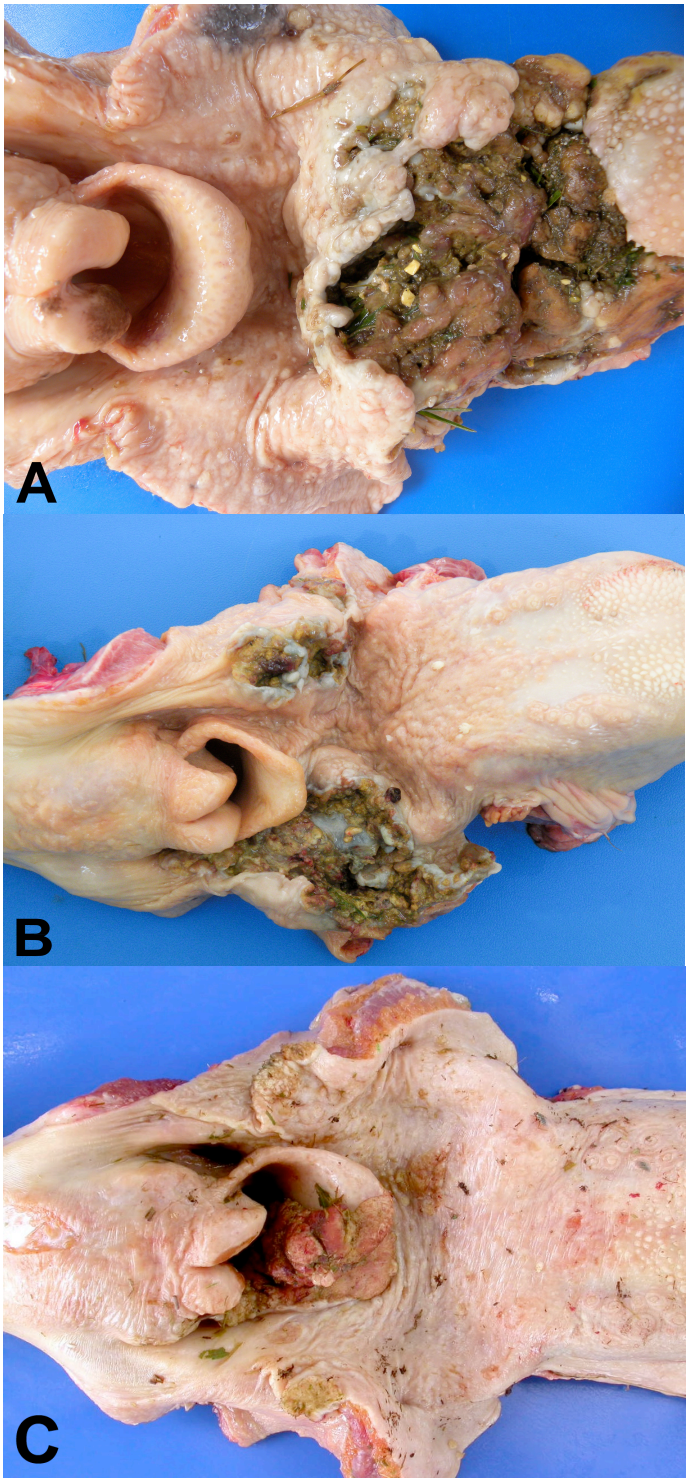

Fig. 1 – Gross appearance of squamous cell carcinomas of the upper digestive tract in cattle grazing on bracken fern. A) Base of tongue. B) Oropharynx. C) Epiglottis.