Clinical, pathological, immunohistochemical and molecular characterization of feline chronic gingivostomatitis

Veronica Machado Rolim1, Saulo Petinatti Pavarini1, Fabrício Souza Campos2, Viviam Pignone3, Cláudia Faraco3, Marcelo de Souza Muccillo3, Paulo Michel Roehe2, Fernanda Viera Amorim da Costa4 and David Driemeier1

Abstract

Objectives This study presents the clinical, pathological, immunohistochemical and molecular characterization of 27 cats with feline chronic gingivostomatitis (FCG).

Methods Oral mucosal biopsies, blood and swabs were collected from cats presenting with oral lesions. The tissue sections were submitted for histopathology and immunohistochemical analysis for feline calicivirus (FCV), feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV). The swabs were subjected to PCR analysis for FCV, and blood for FeLV and FIV.

Results The main clinical findings were dysphagia (83.3%), halitosis (72.2%), sialorrhea (44.4%), weight loss (38.8%), intense oral discomfort (33.3%), oral hemorrhage (22.2%), lackluster and fragile coat (11.1%), and dyspnea (5.5%). Gross inspection revealed bilateral lesions across the palatoglossal fold to the lateral tongue base. The lesions were diffuse, proliferative, intensely red and friable, and bled easily upon examination in 77.8% of cases. In 22.2% of cases, the lesions were multifocal to coalescent, at times forming multiple vesicles on a reddened, edematous palatoglossal fold. Microscopic examination showed that 14.8% of lesions had moderate (grade 2) and 85.2% had severe (grade 3) inflammation. Immunohistochemistry revealed the presence of FeLV antigens in the epithelium and the inflammatory infiltrate of 29.6% of the cats with FCG. FIV antigens were identified in the inflammatory infiltrate of one cat. FCV antigens were not detected in the FCG lesions.

Conclusions and relevance The FCG cases analyzed could not be correlated with FCV. It is possible that FeLV plays a role as causal agent of lesions in cases where the presence of the virus has been confirmed by immunohistochemistry in epithelial samples.

Accepted: 2 January

Introduction

Feline chronic gingivostomatitis (FCG) is a condition often observed in clinical veterinary practice. Characterized by typically bilateral, ulcerous oral lesions, FCG causes intense oral discomfort in affected animals, leading to dysphagia, anorexia and weight loss.1–6

The diagnosis of FCG is based on clinical signs and histopathological examination of the oral lesions, which normally present an intense infiltrate formed essentially by plasma cells and lymphocytes.1–3,7 Believed to be etiologically multifactorial, FCG affects the feline immune system and has been associated with infectious and non-infectious...
agents. However, the increased T-lymphocyte counts compared with B-lymphocyte numbers suggests that FCG may, in fact, be associated with viral infections.

Several infectious agents, such as feline calicivirus (FCV), feline herpesvirus (FHV)-1, feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), along with a wide variety of bacteria, have been investigated in cats with FCG. Nevertheless, all infectious agents cited have been isolated not only from FCG-affected, but also from healthy animals, which results in a less consistent causal relationship.

In the light of the difficulties in establishing associations between FCG and a particular infectious agent, the treatment of the disease poses considerable challenges to clinical practitioners because the lesions often do not respond positively to treatment. The present study reports findings on cases of FCG in cats from Southern Brazil, based on the investigation of clinical signs and the inspection of macroscopic and microscopic lesions. In addition, immunohistochemical (IHC) analysis and PCR were conducted to identify the main suspected viral agents in FCG cases.

Materials and methods

Sample collection

Oral mucosal samples were collected from cats routinely examined at the veterinary hospital of the Federal University of Rio Grande do Sul (UFRGS), Brazil, and from private veterinary clinics in the city of Porto Alegre, state of Rio Grande do Sul, Brazil. All of the animals had oral lesions and were referred for a comprehensive oral health assessment and treatment between March 2012 and October 2013. Oral swabs and 3.0 ml whole blood were collected. The blood samples were obtained from the jugular vein in EDTA-coated tubes. The blood and swab samples were stored at –70°C. All procedures were evaluated and approved by the animal ethics committee of UFRGS (project number 22962).

Breed, sex, age, clinical signs and the clinical progression of FCG were recorded, sorted and analyzed. When authorized by the owners, the cats that died during the study underwent necropsy and routine histology.

Clinical examination

During surgical procedures, the whole oral cavity of the animals was examined, and the lesions were sorted according to severity, as follows: grade 0, absence of lesions; grade 1, mild gingivitis; grade 2, moderate gingivitis; grade 3, severe gingivitis associated with dental calculus; grade 4, gingivitis (with or without calculus) associated with proliferative and/or ulcerous lesions in the palatoglossal fold and/or mucosa and/or tongue (extra-gingival lesions). Only the cases diagnosed as grade 4 lesions were considered FCG and included in this study.

Sample processing and histologic evaluation

The samples were collected in transition areas, which included the lesion areas and normal areas. The oral samples were immediately fixed in 10% buffered saline, routinely processed for histologic investigation, paraffin embedded, cut to 3.0 μm sections and stained using hematoxylin and eosin.

Lesions classified as grade 4 by macroscopic inspection were also divided into four severity grades by microscopy, as follows: grade 0, normal mucosa; grade 1, mild inflammation; grade 2, moderate inflammation; grade 3, severe inflammation.

The presence of metachromatic granules from mast cells was evaluated using toluidine blue. The lesions were then classified again, in four grades: grade 0, less than one mast cell per 10 high-power fields (hpf); grade 1, 1–3 mast cells per 10 hpf; grade 2, 4–8 mast cells per 10 hpf; grade 3, more than eight mast cells per 10 hpf.

IHC evaluation

The tissue sections underwent IHC evaluation for FCV, FeLV and FIV. The monoclonal antibodies used for IHC are listed in Table 1. The previously confirmed positive controls were analyzed simultaneously with the tested samples. The negative controls included tissue samples incubated with phosphate-buffered saline instead of primary antibody.

DNA extraction of whole blood, and PCR for FIV and FeLV

Segments of the proviral genome were detected by PCR, as described by Castro et al., using primers to amplify a conserved region of the gag gene of FeLV (FeLVF 5'-AACCTACCCATACCTCGCCGC-3' and FeLVR 5'-GTTTGTTGTTGTTGTTG-3') and the gag gene of FIV (FIVF 5'-AAAAATGATGTTCAATTTTTATGG-3' and FIVR 5'-CATTCTGTTTGTGTCT-3'). The primers used in the PCR assays were designed to avoid annealing to endogenous retroviral sequences.

RNA extraction from oral swab samples and PCR for FCV

The extraction of RNA from swab samples was carried out using the PureLink Viral RNA/DNA Mini Kit (12280-050) according to the manufacturer’s instructions (ThermoFisher Scientific). The FCV PCR described above was used to detect the viral genome with primers that amplify ORF2, which codes for the main capsid protein, and produced a 955-base pair amplicon.

The PCR products were electrophoresed in 1.5% agarose gels stained with ethidium bromide and inspected under ultraviolet light. A commercial attenuated live vaccine for FCV, FHV-1 and Chlamydothila felis (Felocell CVR-C; Pfizer Animal Health) was used as a source of...
antigen control to standardize the FCV PCR protocol and as a positive control in all tests.

Results

Samples were collected from 43 cats, of which 11.6% (5/43) had grade 1 lesions (mild gingivitis), 9.3% (4/43) had grade 2 lesions (moderate gingivitis) and 18.6% (8/43) had grade 3 lesions (severe gingivitis associated with periodontitis). The remaining cases (60.5%; 26/43) had grade 4 lesions (FCG).

Evaluation of patients

Of the 26 cats included, 84.6% (22/26) were mixed breed, 7.7% (2/26) were Siamese and 7.7% (2/26) were Maine Coons. Sixty-nine percent (18/26) were males, and 31% (8/26) were female. Age was available for 74% of the cases (20/26), as follows: 10% (2/20) were aged between 1 and 3 years; 20% (4/20) between 4 and 6 years; 30% (6/20) between 7 and 9 years; 15% (3/20) between 10 and 12 years; and 25% (6/20) were older than 13 years of age. The animals for which age was not available were adult and mature adult cats. The mean age of the animals was 8.8 years.

Clinical evaluation

The clinical signs were recorded for 65.3% (17/26) of the cases. Eighty-eight percent (15/17) of the cats were dysphagic when consuming dry food (even though they apparently were hungry), 76.5% (13/17) had halitosis, 47.1% (8/17) exhibited sialorrhea, 41.2% (7/17) lost weight, 35.3% (6/17) demonstrated intense oral discomfort upon inspection of the oral cavity during examination, 17.6% (3/17) had occasional oral hemorrhage and the owners of 11.1% (2/18) of the cats reported noticing that the coat of their cat had lost shine.

Gross inspection

Gingivostomatitis lesions were diagnosed bilaterally on the palatoglossal fold, reaching the sides of the tongue base. In 80.8% (21/26) of cases, these reddened lesions were scattered, proliferative, friable and tended to bleed upon palpation (Figure 1a). Additionally, 23.1% (6/26) of the lesions were multifocal or even coalesced, forming several vesicles on the reddish, edematous palatoglossal fold (Figure 1b). In 42.3% (11/26) of cases, intense dental calculi were observed concomitantly with gingivostomatitis. Premolars and molars had been removed previously in 19.2% (5/26) of the animals. Extensive focal ulcerations were observed on the dorsal medial region of the tongue in 11.5% (3/26) of the cats examined (Figure 1c). In addition, gingival ulcers were detected around the upper canines in 7.7% (2/26) of the animals (Figure 1d).

Microscopic examination

Marked hyperplasia, parakeratosis and vacuolar degeneration of the epithelium, apart from moderate amounts of infiltrate, were either focal or multifocal and formed mainly by plasma cells and diverse amounts of lymphocytes, mast cells, Mott cells and neutrophils, especially in ulcerated areas in the submucosa, were detected in 15.4% (4/26) of the animals.

Marked hyperplasia, parakeratosis and vacuolar degeneration of the epithelium were observed in 84.6% (22/26) of the cats examined. These animals also had a discernible diffuse, intraepithelial interface infiltrate formed mainly by plasma cells and variable amounts of lymphocytes, mast cells, Mott cells and neutrophils (Figure 2a). Neutrophils were observed mostly in ulcerated areas (grade 3). Of these animals, 54.5% (12/22) exhibited intense ulcerations in the outer epithelium (Figure 2b), 50.0% (11/22) showed multifocal ulceration areas and in 9.1% (2/22) the oral mucosa was healthy. In 36.4% (8/22) of the cases, the infiltrate in the submucosa was noticed, particularly around vessels and salivary ducts. Granulation tissue was detected in 9.1% (2/22) of the cats.

Toluidine blue staining showed marked mast cell infiltration (grade 3) in 15.4% (4/26) of cases. Moderate (grade 2) and mild (grade 1) infiltration were detected in 38.5% (10/26) and 34.6% (9/26) of samples, respectively.

---

Table 1 Primary antibodies and immunohistochemical protocols applied in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Code number</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Detection method</th>
<th>Chromogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse antifeline immunodeficiency virus (p24 gag)</td>
<td>MCA 2278 (Serotec)</td>
<td>40 mins/100°C, 0.01 M citrate buffer pH 6.0</td>
<td>1:100</td>
<td>LSAB-AP</td>
<td>PR</td>
</tr>
<tr>
<td>Mouse antifeline calicivirus (FCV1-43)</td>
<td>MCA 1897 (Serotec)</td>
<td>40 mins/100°C, Tris-EDTA buffer pH 9.0</td>
<td>1:500</td>
<td>LSAB-AP</td>
<td>PR</td>
</tr>
<tr>
<td>Mouse antifeline leukemia virus (gp70)</td>
<td>FCV2-16 (Custom Monoclonals International)</td>
<td>20 mins/37°C P- XIV</td>
<td>1:50</td>
<td>MACH 4</td>
<td>AEC</td>
</tr>
</tbody>
</table>

P- XIV = Protease XIV (Sigma); LSAB-AP = streptavidin biotin alkaline phosphatase (Dako); MACH 4 = Universal HRP-Polymer (Biocare); AEC = 3-amino-9-ethylcarbazole (Dako); PR = Permanent Red (Dako)
Absence of infiltration, or rare mast cells, were observed in 11.5% (3/26) of samples. Mast cells were located mainly in the submucosa, although intraepithelial mast cell infiltrates were observed in 73.1% (19/26) of animals.

**IHC analysis**
Anti-FeLV and anti-FIV IHC results are shown in Table 2. Marked anti-FeLV immunostaining was noticed in the cytoplasm of epithelial cells (often around the cytoplasm), in the nucleus and cytoplasm of the lymphoplasmacytic infiltrate, and in occasional macrophages in 30.8% (8/26) of animals (Figure 2c,d). No anti-FCV and anti-FIV immunostaining were observed in the oral lesion samples analyzed.

**FIV, FeLV and FCV PCR**
FIV and FeLV PCR results are described in Table 2. Fifteen percent (4/26) of the cases were positive for FIV, and 34.6% (9/26) were positive for FeLV. Co-infection was diagnosed in two animals. No FCV amplification products were obtained in the 26 oral swab samples analyzed.

**Discussion**
The clinical and pathological findings observed in the cats examined in the present study were similar to those reported in previous research on FCG.1–7,9,13,19

In the present study, male cats were more often affected by FCG, although the condition does not have any gender predisposition.1,2,20 One of the possible explanations for the higher incidence of FCG in male cats lies in the more aggressive behavior of these animals compared with females, which increases the possibility of contact with infectious agents such as FIV and FeLV.21

As reported in other studies, FCG has been diagnosed in cats of all age groups.1–3,7,9,13 The samples examined in the present study were collected from adult cats (mean age 8.8 years).22 A recent study showed that older cats as well as younger cats (1–2 years of age) have more severe lesions.9 FCG is believed to affect young cats around the
age of 2 years with mild lesions that evolve into more severe and generalized manifestations.\(^1\)

Halitosis, sialorrhea, difficulty feeding on dry food and a decrease in body condition score were the clinical signs most often observed in the animals examined in the present study. These signs prompted the owners to look for veterinary assistance with their cats, and the symptoms were quite similar to FCG manifestations reported in the literature.\(^1^{-7,9,13,19}\) The owners reported noticing that their cat’s fur looked greasy, lackluster and frail, possibly due to oral discomfort when grooming.

Bilateral, diffuse, proliferative, friable and intensely red lesions on the palatoglossal fold and reaching the sides of the tongue base were the most frequent manifestations in the animals examined. These lesions bled easily upon examination, similarly to previous

**Table 2** Presentation of whole blood PCR and immunohistochemistry (IHC) results of oral biopsies for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) with feline lymphoplasmacytic gingivostomatitis

<table>
<thead>
<tr>
<th>Result</th>
<th>FIV (n = 26)</th>
<th>FeLV (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>PCR 4 (15.4)</td>
<td>IHC 0 (0)</td>
</tr>
<tr>
<td>Negative</td>
<td>PCR 22 (84.6)</td>
<td>IHC 26 (100)</td>
</tr>
</tbody>
</table>

Data are n (%)
Lesion grade 3 (most severe) was the most prevalent in the samples analyzed, according to the classification system proposed by Harley et al. These lesions were characterized by a marked interface infiltrate that blocked the mucosa–submucosa transition zone. The infiltrate was composed mainly of the plasma cells and lymphocytes typically observed in FCG, which is why this form of gingivostomatitis is known as ‘feline lymphoplasmacytic gingivostomatitis’. The ulceration in the epithelium in the samples examined in the present study is often reported in FCG and is normally associated with larger neutrophil infiltrates. The microscopic lesions detected in the samples analyzed were quite similar to the findings reported by Harley et al, because grade 3 lesions were the most prevalent (85.6%) in both studies.

The toluidine blue staining revealed the presence of mild-to-moderate amounts of mast cells, distributed mainly in the submucosa and in the oral epithelium. For Arzi et al., increased mast cell counts are observed in FCG, periodontal disease and tooth resorption in cats. The authors also suggest that mast cells may play a role in the pathogenesis of inflammation in the oral cavity in felines.

Quimby et al. did not find any correlation between FCV, FHV-1, FeLV, FIV, Bartonella species infection and FCG. Owing to the difficulty in confirming the role of an infectious agent in FCG, the disease has been considered multifactorial, involving the immune system of the animals, as well as infectious and non-infectious agents. In the present study, FeLV was the most prevalent infectious agent, both in IHC (30.8%) and PCR (34.6%) protocols. In a prevalence study conducted in Brazil, 11.5% of cats were positive for FeLV; however, in the present study the prevalence of FeLV was higher, probably because the analyzed feline population was composed only of sick animals. Nevertheless, previous studies revealed that the confirmed prevalence of FeLV in feline populations with FCG is rather low, although rates of co-infection with FIV are higher. The IHC evaluation of FeLV confirmed the presence of viral antigen in the epithelium and in the inflammatory infiltrate analyzed. FeLV replication occurs in inflammatory cells, mainly lymphocytes; however, research has confirmed that the virus also replicates in skin epithelial cells, where it causes dermatosis, and in the cornea. In this sense, it is possible that the FeLV plays a role in the etiology of gingivostomatitis; however, more studies are needed to clarify the presence of viral antigens in the injured epithelium.

In this study, no anti-FIV immunostaining was observed, and PCR identified FIV in four animals. It has been reported that the prevalence of FIV is higher in populations with FCG and that lesions are more severe in animals that host the virus. Tenorio et al also revealed that cats co-infected with FIV and FCV have a higher prevalence of oral lesions and that these lesions were more prevalent and severe. Research has shown that people with HIV often have chronic gingivostomatitis associated not only with immunodeficiency, but also with the direct presence of the virus.

Neither the PCR nor the IHC protocols detected the presence of viral agents or amplified the FCV genome in the oral lesions of the cats analyzed in this study. Knowles et al reported that the prevalence of the virus is higher in cats with FCG, where rates reach 92%, than in control animals. Additionally, Addie et al. demonstrated the cured of FCG in a cat when a new round of analysis proved that the lesions did not host the virus anymore. Nevertheless, cats infected only with FCV did not exhibit a higher occurrence or severity of the lesions. The virus has been identified in the oral cavity of cats with FCG, as well as in healthy animals, making it difficult to correlate the presence of the pathogen and the incidence of the disease. In a study that identified FCV and FHV-1 in feline populations in the state of Rio Grande do Sul, Brazil, the prevalence of FCV was higher in healthy individuals than in those with clinical signs. The oral biopsies removed for investigation represent a small portion of the affected region, which means that the virus may not distribute equally across the lesion, therefore inducing false-negative results. Apart from this, it is possible that FCV initially replicated in the epithelium, causing an early lesion that led to oral inflammation, even though the virus was not replicating in the tissue at the moment the material was collected. Similar to IHC, the negative PCR results for the presence of the FCV should be considered with caution because the viral RNA of FCV is rather sensitive to the environment and therefore is easily degraded, inducing false-negative results. Moreover, FCV does not induce a persistent infection such as the feline retroviruses FIV and FeLV, which explains the difficulty in detecting the virus in the population examined in the present study.

Conclusions
The results of the present study show that FCG is an important oral disease in cats. It was not possible to establish FCV as the cause of the disease. It is possible that FeLV plays a role as a causal agent of lesions in cases where the presence of the virus has been confirmed by IHC in epithelial samples.
Conflict of interest  The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding  This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References