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ISOLAMENTO E SOROLOGIA DE CALICIVÍRUS E HERPESVÍRUS FELINO NO RIO GRANDE DO SUL E CARACTERIZAÇÃO MOLECULAR DOS ISOLADOS DE CALICIVÍRUS

TESE DE DOUTORADO

Andréia Henzel

Santa Maria, RS, Brasil

2012

ISOLAMENTO E SOROLOGIA DE CALICIVÍRUS E HERPESVÍRUS FELINO NO RIO GRANDE DO SUL E CARACTERIZAÇÃO MOLECULAR DOS ISOLADOS DE CALICIVÍRUS

por

Andréia Henzel

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiologia da Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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Elaborada por
Andréia Henzel

Como requisito parcial para obtenção do grau de
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RESUMO
Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

ISOLAMENTO E SOROLOGIA DE CALICIVÍRUS E HERPESVÍRUS FELINO NO RIO GRANDE DO SUL E CARACTERIZAÇÃO MOLECULAR DOS ISOLADOS DE CALICIVÍRUS
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Santa Maria, 2 de março de 2012.

O calicivírus felino (*feline calicivirus* – FCV) e o herpesvírus felino tipo 1 (*felid herpesvirus type 1 – FeHV-1*) são os principais agentes das doenças do trato respiratório dos felinos. Ambos os vírus são mundialmente distribuídos, mas no Brasil sua distribuição e prevalência são ainda pouco conhecidas. Na presente tese descrevemos o isolamento e sorologia do FCV e do FeHV-1 em alguns municípios do estado do Rio Grande do Sul (RS), Brasil; e a caracterização molecular do gene da proteína do capsídeo de isolados de FCV. No *Capítulo 1*, descreve-se o isolamento do FCV e do FeHV-1 por meio da coleta de suabes (conjuntival, nasal, oral e orofaringeano) de 302 gatos domésticos. O material coletado foi inoculado em cultivo celular de origem felina (CRFK) e submetidos a PCR e RT-PCR para confirmação em FeHV-1 e FCV, respectivamente. Dos 302 gatos analisados, 55 (18,2%) foram positivos no isolamento; e quando testados por PCR, 29 dos 55 gatos (52,7%) excretavam o FCV, 21 (38,2%) excretavam o FeHV-1 e 5 gatos (9,1%) apresentavam ambos os vírus. Além dessa descrição, realizou-se a padronização de um isolado de FCV e de FeHV-1, para serem utilizados como controle nas técnicas de PCR e na vírus neutralização [VN] (técnica sorológica utilizada no capítulo 3 da tese). O SV65/90 (FCV) e o SV534/00 (FeHV-1) foram os isolados definidos como vírus-padrão. No *Capítulo 2*, incluímos o estudo sobre a análise molecular de 13 isolados de FCV; dez isolados foram obtidos a partir do material descrito no capítulo 1, dois estavam armazenados no laboratório de virologia da UFSM e um foi obtido de um gato acometido pela síndrome gengivo-estomatite. A região analisada foi o gene da proteína do capsídeo, codificado pela ORF2 (*open reading frame*), na qual se localizam as regiões imunodominantes do FCV. A ORF2 foi analisada em nível de nucleotídeos e aminoácidos; e uma análise filogenética dos 13 isolados brasileiros de FCV, incluindo dez cepas de referência e o vírus dos leões marinhos de San Miguel tipo 1 (*San Miguel sea lion virus type 1* – SMSV-1) como um *outgroup,* foi também realizada. Os *primers* foram desenhados com base na sequência completa do genoma da cepa de referência a FCV-F9. Quando os 13 isolados brasileiros de FCV foram comparados a cepa F9, detectou-se grande diversidade molecular nas regiões variáveis do gene e nos epitopos lineares mapeados. O *Capítulo 3* contem um estudo sorológico contra FCV e FeHV-1 em amostras de soro de felinos domésticos. Das 630 amostras testadas pela VN, 53,6% (338/630) foram positivas para um ou ambos os vírus; sendo a prevalência de anticorpos neutralizantes contra o FCV de 39,2% e do FeHV-1 de 30,6%. Os resultados aqui apresentados demonstram a circulação do FCV e do FeHV-1 na população de gatos domésticos analisados, assim como, a presença de gatos portadores para ambos os vírus. Além disso, a caracterização molecular do FCV demonstrou a grande variabilidade genética dos isolados brasileiros em comparação às cepas vacinais e às de referência.
Palavras-chave: FCV, FeHV-1, ORF2, anticorpos neutralizantes, epidemiologia, filogenia.

ABSTRACT
Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

ISOLATION AND SEROSURVEY OF FELINE CALICIVIRUS AND HERPESVIRUS IN THE RIO GRANDE DO SUL STATE AND MOLECULAR CHARACTERIZATION OF ISOLATES OF CALICIVIRUS
AUTHOR: ANDRÉIA HENZEL
ADVISER: RUDI WEIBLEN
Santa Maria, March 2nd, 2012.

The feline calicivirus (FCV) and the felid herpesvirus type 1 (FeHV-1) are the main agents of the respiratory tract diseases of felines. Both viruses are distributed worldwide, however its distribution and prevalence in Brazil are not well known. In the present thesis we describe the isolation and one serosurvey of FCV and FeHV-1 in some counties of the Rio Grande do Sul State, Brazil; and the molecular characterization of the capsid protein gene of FCV isolates is also described. In *Chapter 1*, we describe the epidemiologic survey of FCV and FeHV-1 through investigation of the conjunctival, nasal, oral and oropharyngeal swabs from 302 domestic cats. The viral isolation was performed in Crandell-Reese feline kidney cells and the isolates were submitted to PCR and RT-PCR for confirmation of the presence of the FeHV-1 and FCV, respectively. Fifty five (18.2%) of the 302 cats analyzed were positive for the viral isolation; when tested by PCR, 29 cats (52.7%) were shedding the FCV, 21 (38.2%) shedding FeHV-1, and 5 cats (9.1%) shedding both viruses. In addition, isolates of FCV and FeHV-1 were standardized to be used as control in the PCR and virus neutralization (VN) assays (serological technical used in chapter 3 of the thesis). The SV65/90 (FCV) and the SV534/00 (FeHV-1) isolates were defined as standard viruses for the assays. In *Chapter 2*, the molecular characterization of 13 FCV isolates is described; ten isolates came from the survey performed in chapter 1, two were deposited in the virology laboratory of the UFSM and one was obtained from a cat with gingivitis-stomatitis syndrome. The ORF2 (open reading frame) region that codifies the major protein of the viral capsid was sequenced, which includes an immunodominant region of the virus. The ORF 2 was analyzed at the nucleotide and amino acid levels and these data was used for phylogenetic studies of the 13 Brazilian isolates of FCV. These isolates were compared to ten reference strains of FCV and to the San Miguel sea lion virus type 1 (SMSV-1) as an outgroup in phylogenetic study. The primers were designed using the complete sequence of FCV-F9. The comparison of the 13 Brazilian isolates with the F9 strain revealed a large molecular diversity among them, concentrated mainly along the linear epitopes of the region. The *Chapter 3* describes a serosurvey against FCV and FeHV-1 in serum samples from domestic feline. From the 630 samples tested by the VN assay, 53.6% (338/630) were positive to one or both viruses with a prevalence of 39.2% for neutralizing antibodies against FCV and 30.6% for FeHV-1. The results from the present study demonstrated that the FCV and FeHV-1 are circulating among the feline population examined, and also, the presence of carriers of the viruses among these cats. Besides, the molecular characterization of the FCV evidenced the great genetic variability of the Brazilian isolates when compared to vaccine and reference strains.

Keywords: FCV, FeHV-1, ORF2, neutralizing antibodies, epidemiology, phylogeny.

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LISTA DE ABREVIATURAS E SIGLAS

Ac – anticorpo

bp – par de bases (*base pair*)

conE – região conservada da região E (*conserved E*) - ORF2 do FCV

CRFK – linhagem celular de rim de felino (*Crandell-Reese feline kidney*)

FCV – calicivírus felino (*feline calicivirus*)

FeHV-1 – herpesvírus felino tipo 1 (*felid herpesvirus type 1*)

FITC – isoticionato de fluoresceína (*fluorescein isothiocyanate*)

HVR – região hipervariável (*hypervariable region*)

fJAM-1 – *feline Junctional Adhesion Molecule 1 receptor*

IFA – imunofluorescência (*immunofluorescence*)

IgG – imunoglobulina de classe G

kb – kilobases

ME – microscopia eletrônica

nm – nanômetros

ORF – fase aberta de leitura (*open reading frame*)

PCR – reação em cadeia da polimerase (*polymerase chain reaction*)

RT-PCR – transcrição reversa - reação em cadeia da polimerase (*reverse transcription - polymerase chain reaction)*

SMSV-1 – vírus dos leões marinhos de San Miguel tipo 1 (*San Miguel sean lion virus type 1*)

SV – Setor de Virologia

TK – timidina quinase (*thymidine kinase*)

UFRGS – Universidade Federal do Rio Grande do Sul

UFSM – Universidade Federal de Santa Maria

UPF – Universidade de Passo Fundo

URI – infecção respiratória superior (*upper respiratory infection*)

URTD – doença do trato respiratório superior dos felinos (*upper respiratory tract disease*)

VN – vírus neutralização

VP – proteína viral (*viral protein*)

VSD-FCV – doença virulenta sistêmica associada ao FCV (*virulent systemic disease associated – FCV*)

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ANEXO 1

SUMÁRIO

1. INTRODUÇÃO

As doenças respiratórias infecciosas dos felinos se constituem em um problema clínico frequente e de grande importância na medicina dos felinos (RADFORD et al., 2009; THIRY et al., 2009). O calicivírus felino (*feline calicivirus* – FCV) e o herpesvírus felino tipo 1 (*felid herpesvirus type 1* – FeHV-1), juntamente com a *Bordetella bronchiseptica, Chlamydophila felis* e *Mycoplasma felis*, são os principais agentes da doença do trato respiratório superior dos felinos, conhecida como URTD (*upper respiratory tract disease*) ou URI, infecção respiratória superior (*upper respiratory infection*); no entanto, as bactérias são considerados agentes secundários à infecção pelo FCV e/ou FeHV-1 (GASKELL & KNOWLES, 1989). Na síndrome URTD, ambos os vírus tem sido considerados de igual importância, mesmo havendo um maior isolamento do FCV em comparação ao FeHV-1 (GASKELL & KNOWLES, 1989; HARBOUR et al., 1991; BINNS et al., 2000).

Além do envolvimento do FCV e do FeHV-1 com a URTD, gatos infectados por ambos os vírus podem apresentar infecções subclínicas; e para o FCV: faringites; a severa doença oral crônica conhecida por complexo gengivo-estomatite linfoplasmocítica (*lymphoplasmacytic gingivitis stomatitis* – LPGS); manqueira ou claudicação; e a síndrome sistêmica altamente virulenta conhecida como VSD-FCV (*virulent systemic disease associated* - FCV) também são descritos (BENNETT et al., 1989; PEDERSEN et al., 2000; PESAVENTO et al., 2008; GASKELL et al., 2007; RADFORD et al., 2007, 2009). Entretanto, o isolamento viral em gatos clinicamente saudáveis é comumente reportado, pois, para ambos os vírus, gatos recuperados clinicamente após infecção aguda podem tornar-se portadores (GASKELL et al., 2007; RADFORD et al., 2007). Gatos portadores do FCV podem excretar o vírus continuamente (semanas, meses e até anos); e os gatos portadores do FeHV-1, o excretam periodicamente após reativação da infecção latente em situações de *stress* como: transporte, mudança de *habitat,* lutas entre machos, parição e lactação (GASKELL et al., 2007; GASKELL & POVEY, 1977; RADFORD et al., 2007).

O FCV é um vírus RNA de cadeia simples, polaridade positiva, não envelopado, pertencente ao gênero *Vesivirus* da família *Caliciviridae* (RADFORD et al., 2007). O tamanho do genoma do FCV é de aproximadamente 7,7 kb, e codifica três ORFs (*open reading frames*).

(GREEN et al., 2000). A ORF 1 codifica as proteínas não-estruturais, incluindo a protease viral e a RNA polimerase (RADFORD, et al., 2007); a ORF 2 codifica a principal e maior proteína do capsídeo, VP1 [*viral protein*] (SEAL et al., 1993), e a ORF 3 codifica a menor proteína estrutural do vírus, VP2 (SOSNOVTSEV & GREEN, 2000) [ver Figura 1 do Capítulo 2, página 54].

A ORF2 é dividida em seis regiões (nomeadas de "A a F") com base em sequências conservadas (NEIL, 1992; SEAL et al., 1993). A região E foi ainda dividida em duas regiões hipervariáveis (*hypervariable* – HVR): 5'HVR_E e 3'HVR_E; separada por uma região central conservada [conE] (SEAL et al., 1993; 1994). As regiões HVR_E contêm epitopos para os linfócitos B, o que faz dessa região ser alvo para anticorpos neutralizantes; assim como, pode ter função de evasão imune viral durante a infecção persistente no hospedeiro (TOHYA et al., 1997; GEISSLER et al., 2002; RADFORD et al., 2007). Epitopos conservados tem sido identificados dentro das regiões D e conE, mas seu papel na indução de anticorpos ainda é desconhecido (RADFORD et al., 1999).

Como todo vírus RNA, o FCV possui altas taxas de mutação no genoma, devido à falta de correção do erro (*proofreading*) da polimerase viral durante sua replicação (DOMINGO et al., 1997). Essas mutações ocorrem tanto "*in vivo*" quanto "*in vitro*", resultando em novos variantes virais, os chamados *quasispecies* (RADFORD et al., 1998). Assim, o FCV pode responder rapidamente às pressões de seleção ambiental e imune do hospedeiro, dificultando o controle da infecção (RADFORD et al., 1998; 2007). Essa diversidade genética pode provavelmente explicar as amplas variações nas síndromes clínicas, a pouca eficácia das vacinas, a dificuldade de induzir proteção vacinal cruzada e também pode contribuir para a infecção persistente no hospedeiro (LAURITZEN et al., 1997; KREUTZ et al., 1998; GLENN et al., 1999; RADFORD et al., 2007). Além disso, os isolados altamente virulentos da síndrome sistêmica (VSD-FCV) são outra consequência dessa diversidade genética e antigênica do FCV (PEDERSEN et al., 2000). No entanto, mesmo com essa ampla variabilidade em nível molecular, todos os isolados de FCV pertencem ao mesmo biotipo, genótipo e sorotipo (GEISSLER et al., 1997; POULET et al., 2000; RADFORD et al., 2007)

O FCV é geralmente transmitido por contato direto entre gatos doentes ou persistentemente infectados (POVEY & JOHNSON, 1970; RADFORD, et al., 2007; 2009). O vírus penetra no organismo pela via oronasal, conjuntival e orofaringeal, sendo este último, definido como o sítio primário da replicação do FCV. A transmissão indireta também ocorre,

especialmente dentro de gatis onde os animais estão próximos. Nesses ambientes as secreções podem contaminar gaiolas, utensílios de limpeza e de alimentação, ou mesmo uma pessoa pelas mãos e roupa podem carrear o vírus (RADFORD et al., 2007). O FCV pode permanecer infeccioso por até um mês em superfícies secas a temperatura ambiente, e por períodos maiores em condições de temperatura mais baixa e úmida (CLAY et al., 2006).

O FeHV-1, o agente da rinotraqueíte viral felina (FVR) (CRANDELL, 1971), é um vírus pertencente ao gênero *Varicellovirus,* subfamília *Alphaherpesvirinae* da família *Herpesviridae* (GASKELL et al., 2007). O FeHV-1 possui um capsídeo icosaédrico envolto por envelope (GASKELL et al., 2007), sendo relativamente frágil no ambiente externo e altamente susceptível aos efeitos dos desinfetantes comuns (SCOTT, 1980). O genoma do FeHV-1 é constituído por uma fita dupla de DNA com aproximadamente 134 kb (ROTA et al., 1986) e codifica no mínimo cinco glicoproteínas (gp) de superfície (MAES et al., 1984). As gp (s) presentes no envelope dos herpesvírus têm importante papel na indução da imunidade humoral e celular (MAES, et al., 1984; MAEDA, et al., 1998). Os isolados de FeHV-1 podem diferir em virulência, no entanto todos os isolados de FeHV-1 pertencem a um mesmo sorotipo (THIRY, 2006; GASKELL et al., 2007).

A transmissão do FeHV-1 ocorre pelas vias nasais, orais e membranas conjuntivais (GASKELL et al., 2007). A excreção de vírus infeccioso por essas vias, durante o curso da infecção, é de uma a duas semanas para gatos com infecção aguda. Em gatos com infecção inaparente, a duração é de um dia à uma semana e de gatos portadores a excreção é intermitente, durante os episódios de reativação (GASKELL & WARDLEY, 1977). No FeHV-1 assim como em todos os alphaherpesvírus, a infecção primária caracteriza-se por replicação viral citopática nas membranas nasais, conjuntivais e na traquéia (GASKELL et al., 2007; GASKELL & POVEY, 1979; POVEY & JOHNSON, 1970); e pela latência do genoma em tecidos neurais (GASKELL et al., 1985; REUBEL et al., 1993). Após a replicação nos sítios primários de infecção, o FeHV-1 ascende via axônio dos nervos sensoriais, estabelecendo latência no gânglio trigêmeo (MAGGS, 2005).

Os sinais clínicos observados em animais infectados pelo FeHV-1, tanto em condições naturais como experimentais, são: depressão, espirros, inapetência, febre, descarga nasal e ocular serosa (HOOVER et al., 1970; CRANDELL, 1973). Esses sinais podem ser acompanhados por excessiva salivação, conjuntivite, algumas vezes com hiperemia severa e equimoses; e em casos

severos, dispnéia e tosse também podem ocorrer (LOVE, 1971; SHIELDS & GASKIN, 1977). Normalmente, os sinais clínicos se resolvem dentro de 10-20 dias, mas em alguns animais pode resultar em sequelas crônicas tais como conjuntivite e rinite persistente ou intercorrente (GASKELL & WARDLEY, 1977). A conjuntivite pode estar associada com úlceras de córnea, as quais muitas vezes podem evoluir à um quadro crônico. A ceratite estromal, outro quadro clínico observado, é uma reação secundária do tipo imunomediada devido à presença de vírus no epitélio ou no estroma (THIRY et al., 2009). Em alguns casos, dano dos cornetos nasais na doença aguda pode predispor o desenvolvimento de rinite crônica (GASKELL et al., 2007).

A vacinação contra ambos os vírus teve início na década de 70 (GASKELL & KNOWLES, 1989); no entanto, a presença e o envolvimento do FCV e do FeHV-1 com a URTD não foi alterado (HARBOUR et al., 1991). Vacinas vivas ou inativadas, com aplicação parenteral, são comercializadas mundialmente e também no Brasil; e nos Estados Unidos uma vacina viva de administração intranasal contra o FeHV-1 também é comercializada (ORR et al., 1978; 1980; GASKELL et al, 1982; LAPPIN et al., 2006). As vacinas em geral são seguras e efetivas em reduzir ou prevenir as doenças respiratórias/orais clássicas, embora elas não previnam reinfecções e o estado de portador (GASKELL et al., 2007; RADFORD et al., 2007). A maioria das vacinas disponíveis mundialmente utiliza uma cepa amplamente reativa, a FCV-F9, embora outras cepas de FCV sejam comercialmente usadas, tais como a FCV-F7 e FCV-255 (BAULCH-BROWN et al., 1999; RADFORD et al., 2006; OHE et al., 2007). Na Europa, vacinas comerciais se baseiam em duas cepas de FCV, FCV-431 e FCV-G1 (POULET et al., 2005).

Falhas vacinais contra ambos os vírus ocorrem, e podem estar associadas com a preexistência do estado de portador, doença intercorrente e/ou interferência de imunidade passiva (HARBOUR et al., 1991). Entretanto, as falhas vacinais relacionadas ao FCV, provavelmente estão relacionadas à sua diversidade antigênica (BAULCH-BROWN et al., 1999; SCHORR-EVANS et al., 2003). Os isolados associados às falhas vacinais são designados: *vaccine breakdown strains* - VBS (OHE et al., 2007). Estudos relatam que em gatis de reprodução foram isolados vírus relacionados e não relacionados às cepas vacinais (RADFORD et al., 2001). Para determinar se a origem dos vírus isolados de gatos é derivada de vírus de campo ou das cepas vacinais, realiza-se o sequenciamento da região 5'HVR_E do gene do capsídeo [Figura 1 do Capítulo 2, página 54]. Grande parte dos isolados de falhas vacinais foram encontrados como sendo vírus de campo, e que diferem das cepas vacinais em 21,3 – 38% (RADFORD et al.,

1997).

Tanto o FCV quanto o FeHV-1 são mundialmente distribuídos (HARBOUR et al., 1991; BINNS et al., 2000); no entanto no Brasil ainda pouco se conhece sobre sua distribuição e prevalência. O primeiro isolamento do FCV foi descrito em 1988 por Weiblen et al. (1988), e do FeHV-1, durante a execução desse projeto. O FeHV-1 era somente diagnosticado clinicamente pelos veterinários na síndrome clínica da FVR, também chamada de "gripe do gato" (observação do autor).

O que motivou a realização deste trabalho foi a necessidade de se conhecer um pouco mais sobre a distribuição e a prevalência do FCV e do FeHV-1 no estado do Rio Grande do Sul; e a caracterização molecular do FCV. Assim a tese é dividida em três capítulos. No capítulo 1, descreve-se o isolamento do FCV e do FeHV-1 em 302 gatos coletados em alguns municípios do estado do Rio Grande do Sul (RS). O Capítulo 2 contém um estudo molecular de 13 isolados Brasileiros de FCV no qual se analisou a sequência de amino ácidos da proteína do capsídeo (VP1) e a organização filogenética com mais dez sequências de referência do FCV. No capítulo 3 descreve-se um estudo sorológico contra o FCV e o FeHV-1 em 630 amostras de soro felino coletadas em alguns municípios do RS e em maior número, fornecidas pelos laboratórios de patologia clínica de três Universidades do sul do país (UFRGS, UFSM e UPF). Em anexo estão demonstrados os resultados das técnicas da microscopia eletrônica, imunofluorescência do FCV, PCR e o efeito citopático em cultivo celular de origem felina (CRFK) utilizadas para a padronização dos isolados SV65/90 e SV534/00, FCV e FeHV-1 respectivamente. Ambos os isolados foram utilizados como controle positivo na realização das PCRs e como vírus-padrão para a técnica de vírus neutralização.

2. CAPÍTULO 1

Isolation and identification of feline calicivirus and feline herpesvirus in southern Brazil

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Abstract

Feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1) are the two primary causes of upper respiratory tract disease in cats. The aim of this study was to demonstrate the distribution of FCV and FHV-1 among the feline population of several counties in Rio Grande do Sul State, Brazil. To this end, conjunctival and nasal swabs were collected from 302 cats from different locations, including households, breeding catteries, veterinary clinics, animal hospitals and experimental research facilities. The samples were collected between July 2006 to June 2009. The virus isolation was performed in CRFK cells and, subsequently, the identification was confirmed by PCR. FCV, FHV-1, or both were isolated from 55 cats from 28 different locations. FCV alone was isolated from 52.7% (29/55) of the animals that tested positively, FHV-1 alone was isolated from 38.2% (21/55) of the animals that tested positively, and co-infection were detected in 9.1% (5/55) of the animals that tested positively. Virus detection was more prevalent in cats that were less than 1 year old among animals that shared a living space with other cats and females. FCV and FHV-1 were isolated from vaccinated cats. In addition, both viruses were isolated from cats that showed no signs of disease. The results suggest that a carrier state is common for both viruses in the evaluated population. A search for other causes of respiratory disease in that population is necessary; and further studies relating to the molecular characterization of viruses and vaccine efficacy are also necessary.

Key words: FCV, FHV-1, URTD, epidemiology

Introduction

Infectious respiratory disease is a major clinical problem in feline medicine. Such infections are primarily caused by either one or both of two viruses: feline calicivirus (FCV) and

feline herpesvirus type 1 (FHV-1) (11, 25). These viruses have worldwide distribution, and it is estimated that roughly 80 percent of upper respiratory tract disease (URTD) infections in cats are caused by FCV and FHV-1 (12, 17).

FCV is a virus that belongs to the *Vesivirus* genus and the *Caliciviridae* family. It has a small, single-stranded, positive-sense RNA genome that encodes three open reading frames [ORFs] (25). Individual FCV isolates may differ, but they all belong to a single serotype (25). Clinical disease caused by FCV is typically characterized by oral ulcerations with or without mild respiratory and conjunctival signs (25). The virus has also been associated with the presence of transient and shifting lameness, hemorrhagic-like fever (21), abortion (5) and chronic stomatitis (16). Following recovery from acute disease, cats may become a carrier, effectively shedding the virus from the oropharynx (24, 31). The duration of this carrier state is variable and ranges from months to years in individual animals (9). Higher prevalence of FCV has been associated with cats of less than 12 months and households were large number of cats are housed together (2, 3, 30).

More recently, and more worryingly, highly virulent strains of FCV have emerged that are associated with outbreaks of disease with high mortality and a new range of clinical features, FCV- associated virulent disease (VSD) - previously haemorrhagic-like fever (21, 25). In addition to upper respiratory tract disease, the affected cats show to varying degrees pyrexia, cutaneous oedema, ulcerative dermatitis, anorexia and jaundice, with up 50 per cent of them dying or being euthanased in extremis (4). Adult cats are frequently affected more severely than kittens, and vaccination does not appear to be protective (4). FCV can be isolated from oral and conjunctival swabs from affected cats (4). VSD-FCV has not yet been described in Brazil.

FHV-1 is the agent of feline viral rhinotracheitis (FVR) (11, 8). It is a DNA virus that is a member of the *Varicellovirus* genus in the *Alphaherpesvirinae* subfamily (11). Only one serotype

of this virus exists, and like other *alphaherpesvirus*, FHV-1 induces latency in nervous ganglions (11). Thus, clinically recovered cats are carriers that undergo periodic episodes of virus reactivation, particularly after stress (11). In both experimental and natural infections, symptoms include the following: depression, sneezing, inappetence, pyrexia and serous ocular and nasal discharges (11). Cats of any age, sex or breed are susceptible, but a severe syndrome is usually restricted to kittens of up to six months of age (23). It is estimated that more than 90% of the cats are soropositive to FHV; and that a minimum of eighty percent remains latently infected with 45% shedding the virus by all life long (18).

The nasal, oral and conjunctival vias are the natural routes of infection for FCV and FHV-1 (11, 25). Transmission occurs mainly through direct contact between infected and susceptible cats; however, indirect transmission can also occur in the case of FCV, particularly within a cattery where secretions may contaminate cages, feeding and cleaning utensils or personnel (11, 25). Reports from cats with URTD have revealed a prevalence ranging among 20-53% for FCV and 10-34% for FHV-1 (2, 12, 16). In the general healthy cat population from several European countries, USA and Korea, the prevalence of FCV has varied from 15% to 31% (2, 16) whereas values ranging from less than 1% to 63% have been estimated for the prevalence of FHV-1 (2, 13, 15).

These viruses are still prevalent in the feline population despite the fact that vaccination against FCV and FHV-1 has been practiced since the 1970s (2, 29). Vaccinations may have reduced the overall severity of disease; however, in some vaccinated individuals, disease may still occur (11, 25, 29). Commercially available vaccines are generally safe and protect reasonably well against disease, although they do not prevent infection, the shedding of virus or even the development of the carrier state (9, 11, 20, 25). Live attenuated and inactivated vaccines are available (11, 25), and vaccine virus shedding after vaccination has been described

experimentally for a temperature sensitive FHV-1 vaccine (33). In that case, vaccine virus has been shed for 25 days after vaccination (33).

There is little available information about FCV and FHV-1 in Brazil. Vaccination is performed in Veterinary Clinics and Hospitals with live attenuated and inactivated vaccines however the percentage of the population that is actually vaccinated is unknown. The isolation of FCV has been described once in the southern part of the country (32), followed by an experimental study regarding the pathogenicity of the virus (22). However, to our knowledge, there has not yet been a described isolation of FHV-1 in Brazil. Regardless, evidence of the presence of both viruses has been obtained from serologic surveys performed in populations of wild (6, 27) and domestic felines (14). The aim of the present study was to generate insight into the epidemiology of FCV and FHV-1 in the southern part of Brazil. Thus, conjunctival, nasal, oral and oropharyngeal swabs were collected from 302 cats in several counties in Rio Grande do Sul State, and the resulting isolation and identification of FCV and FHV-1 are described.

Material and methods

Source of samples

The samples consisted of conjunctival, nasal and, occasionally, oral and oropharyngeal swabs collected from cats with or without clinical signs of respiratory disease. The samples were collected from July 2006 to June 2009 from 302 cats from veterinary clinics and hospitals, residences, breeding catteries and experimental populations. The samples were collected from animals in the following counties of the Rio Grande do Sul State, Brazil: Cachoeira do Sul, Canoas, Estrela, Nova Palma, Pelotas, Porto Alegre, Santa Maria and Santo Ângelo. All of the animal handling procedures were performed under veterinary supervision and following the recommendations of the Brazilian Committee on Animal Experimentation (COBEA, law #6.638

of May, 8th, 1979). The experiments were approved by an institutional committee on animal welfare and ethics (UFSM - approval number # 61/2009).

Cell culture and virus isolation

The feline kidney cell line CRFK (*Crandell-Reese feline kidney*) was used for virus isolation and amplification. Cells were routinely maintained in Eagle's minimal essential medium (MEM) containing penicillin (1.6 mg/L), streptomycin (0.4 mg/L), amphotericin B (2.0 mg/L), and 10% fetal calf serum.

Swabs were kept in microtubes with MEM medium (0.5 ml) and stored at – 70°C until used in experiments. The swabs were briefly agitated in a vortex and the content was then transferred to microcentrifuge tubes and centrifuged at 10.000 x *g* for 5 min. The supernatants (0.15 ml) were inoculated onto CRFK cell monolayers grown in 24-well plates and were submitted to three passages of five days each while the cells were monitored for cytophatic effect (CPE). Cultures exhibiting CPE were investigated for the presence of feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1), or both viruses using a polymerase chain reaction (PCR) assay. Three blind passages were performed for cultures not exhibiting CPE, and the cultures were considered negative for virus isolation.

Extraction of DNA and RNA, primers and PCR

Only one sample from each animal was tested by PCR and RT-PCR, including those from which the viruses were isolated from different swabs. Different samples isolated from the same cat were pooled to perform the PCR/ RT-PCR. The extraction of FCV RNA and FHV-1 DNA was performed using TRIzol and DNAzol reagents (Invitrogen, Carlsbad, CA, USA), respectively. The extractions were performed from cell cultures inoculated with the respective

viruses following the manufacturer's protocol. After extraction, DNA was solubilized in 8 mM NaOH (0.2 ml) and stored at – 20°C until further testing. The RNA was solubilized in 30 µl of ultra-pure water with DEPC (diethylpyrocarbonate) and stored at -70°C until use. The cDNA was synthesized in 20 µl of total solution containing the following: 2 µl of RNA (approximately 100 ng), 100 ng of random primers, 1X buffer from reverse transcriptase (RT), 25 mM $MgCl_2$, 10 mM dNTPs, 0.1 Mm DTT, 40 U RNaseOUT and 200 U RT (SuperScript™ III RT – Invitrogen). The solution was incubated at 65°C for 5 min, 25°C for 10 min, 42°C for 50 min and 85°C for 5 min. The cDNA was used as a template for the PCR for FCV identification.

The ORF2 (regions B to F), which encodes the major capsid protein (25), was the target region for amplification of the FCV cDNA. The amplified product resulted in a fragment of 955 bp (base pair). The primer sequences were 8F (forward) 5' – CACSTTATGTCYGACACTGA – 3' (position 6142 B region) and 8R (reverse) 5' – CTRGADGTRTGCARRATTT – 3' (position 7097 F region), based on the FCV-F9 strain (GenBank access number M86379). The primers used were degenerate. The letters S, Y, R and D refer to C/G, T/C, A/G, and A/C/T, respectively, and were previously developed (19). The PCR conditions used were as it follows: 94°C for 5 min for the initial denaturation, followed by 35 cycles of three steps of 94°C for 45 s, 48°C for 45 s, 72°C for 45 s, and a final extension of 7 min at 72ºC.

For FHV-1 the thymidine kinase enzyme (TK) gene was the region of the viral DNA amplified by PCR. The primer set used in the reaction was previously developed (28), and the product size is 287 bp (GenBank access number M26660). The primer sequences used were as follows: Herp_F (forward) 5' – GACGTGGTGAATTATCAGC – 3' (position 510 TK gene) and Herp_R (reverse) 5'– CAACTAGATTTCCACCAGGA – 3' (position 797 TK gene). The PCR conditions used were as follows: 94°C for 5 min, followed by 40 cycles at 94°C for 45 s, 56°C for 30 s, 72°C for 45 s and a final extension of 7 min at 72°C.

The resulting PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. A commercial live attenuated vaccine to both viruses plus *Chlamydophila felis*, Felocell CVR-C (Pfizer Animal Health, USA), was used as a control in standardizing the PCR reactions and as a positive control in all of the tests. Two of the samples isolated in our lab and identified by electronic microscopy (EM), SV65/90 (FCV) and SV534/00 (FHV-1) were also used as positive control in the reactions. Negative controls consisted of DNA and RNA extracted from mock-infected CRFK cells.

Results

Samples

A total of 572 swabs samples were collected from 302 domestic felines either with or without clinical signs suggesting infection by feline calicivirus (FCV) and/or feline herpesvirus type 1 (FHV-1). Most of the samples were conjunctival or nasal swabs; only in two cases and one case were the swabs collected from the oral and oropharyngeal cavities, respectively (Table 1). The feline population sampled in this article consisted of household cats living alone, household cats living with other cats, breeding cattery cats, hospital cats, cats in veterinary clinics and cats kept isolated for experimental research. The data were organized according to animal age, gender, vaccination status, habitat, presence or absence of clinical signs and the type of swabs collected (Table 1).

Virus detection and identification

FCV, FHV-1, or both were detected in 55 from the 302 cats examined in this survey (Tables 1 and 2). FCV alone was isolated in 52.7% (29/55) of the cats that tested positively, FHV-1 alone in 38.2% (21/55) and double infection was detected in 9.1% (5/55) [Table 2]. Virus

isolation was confirmed in all cases by PCR and RT-PCR for FHV-1 and FCV, respectively.

Epidemiological and clinical aspect

Virus was isolated from cats showing clinical signs of disease and from healthy cats (Table 1). The opposite also held true, as virus was not detected in samples from cats with evident clinical manifestations of respiratory disease (Table 1). The clinical signs most frequently observed in the 25 cats with evidence of disease were as follows: ocular discharge in 16/25 (64%); nasal discharge in 7/25 (28%); conjunctivitis, sneezing, coughing, dyspnea, fever and anorexia in 6/25 (24%); and oral lesions (ulcer) in 3/25 (12%). Respiratory disease was observed in 24 out of the 25 cats that tested positively for virus isolation, and 2 of them also showed oral ulcers. Only 1 of the 25 cats only showed oral ulcers without other signs of respiratory disease. FCV alone was isolated from the 3 cases in which ulcers were detected.

FCV and FHV-1 were isolated from both vaccinated and non-vaccinated cats. When each situation is considered separately, the results reveal that 15.7% (14/89) of the total population of vaccinated cats tested positively for one or both viruses, whereas approximately 19% (36/186) of the non-vaccinated cats tested positively. A comparison of the data relating vaccine status to the presence or absence of clinical signs did not reveal a substantial difference among the vaccinated and non-vaccinated groups. The analysis demonstrated that 40% of the 25 cats that exhibited clinical signs and from which virus was isolated were vaccinated, whereas 48% were not vaccinated. In addition, among the 30 cats that tested positively but did not show signs of disease, approximately 43% were not vaccinated and 13.3% were vaccinated (data not shown).

The 55 cats that tested positively for viral isolation came from 28 different locations. FCV alone was isolated from 17 (60.7%), FHV-1 alone from 7 (25%), and both viruses in four out of the 28 places (14.3%) [data not shown]. FCV alone was isolated from eight of thirteen cats kept

together for experimental research. Cats living alone in two different locations were positive only for the presence of FCV. The cats from the breeding catteries were found to be only positive for FHV-1. There were six positive cats in each of two catteries and two cats from another breeding cattery. Among the cats from the veterinary hospitals, which came from four different locations, FCV was detected in two locations; both viruses were detected in the samples coming from cats obtained from the other two locations. FCV was isolated from 66.6% (12/18), FHV-1 from 22% (4/18) and both viruses were isolated from 11.1% (2/18) of the samples coming from the remaining eighteen locations sheltering cats living with another cat or cats (data not shown).

With regards to age, approximately 93% of the 55 cats exhibiting positive results for virus isolation were up to 5 years old. Taking into consideration cats from which only one of the viruses or both viruses where isolated, FCV was isolated more often from cats between 1 and 5 years old, whereas FHV-1 was isolated more often from cats under 1 year old (Table 2).

The classification by gender revealed that the difference between the number of samples collected from male and female cats was only four, although the number of females that tested positively for virus isolation was more than two-fold higher the number of males that tested positively [Table 2]. With regards to the origin of the cats, females were positive in 16 locations, males in 9 locations and both male and female in 3 locations (data not shown).

Discussion

The epidemiological conditions of feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1) are known among the feline population worldwide (11, 25). Although it is generally assumed that these conditions are similar in Brazil, no major study regarding these viruses has been performed in that country, with the exception of several serological surveys (6, 14, 27) and an experimental study regarding pathogenicity (22). The diseases of the respiratory tract of

felines are an important and recurrent problem for veterinarians and cat owners globally, and FCV and FHV-1 have been described as one of the primary causes of these clinical manifestations (2, 8). In this study, FCV, FHV-1 or both were isolated and identified from 55 cats with or without clinical signs from a total of 302 animals sampled in some cities of the Rio Grande do Sul State in the southern part of Brazil.

In the present article, FCV was isolated more often, in terms of the overall results, but its frequency was different when the groups were analyzed separately (Table 2). FCV was the primary virus isolated in most of the groups examined, although FHV-1 was the virus most frequently isolated from vaccinated cats and was also isolated slightly more frequently than FCV in cats exhibiting clinical signs of disease. A greater prevalence of FCV in comparison to FHV-1 has been reported in cats with clinical manifestations of disease as well for clinically healthy cats (2, 16), although it has been shown that FHV-1 isolation is generally related to the presence of clinical signs, whereas FCV is not (12). Furthermore, FHV-1 is the virus most commonly identified when respiratory clinical manifestations are observed (7, 13, 16, 34); in the present study, signs of respiratory disease were present in 24 out of the 25 sick cats from which FCV, FHV-1 or both were isolated. Conversely, FCV was present in samples from the three cats showing signs of oral lesions in this study (data not shown), which matches results from other studies that have associated oral ulcers more consistently with FCV infection (25, 26, 34).

The higher prevalence of FCV in comparison to FHV-1 isolated in the United Kingdom has been attributed to vaccines that began to be applied in the 1970s (12). This vaccination likely contributed to the reduction of the number of cats in the population excreting FHV-1 but did not significantly affect the number of cats excreting FCV (12). The biology of the viruses is one characteristic that could contribute to this finding; FCV is an RNA virus with wide genetic and antigenic diversity, whereas FHV-1 is a stable DNA virus (25). In this article, the number of

vaccinated cats excreting FHV-1 was higher than that excreting FCV (Table 2). However, it should be noted that there was a unique situation in this study that could cause such results. Most of the isolated FHV-1 came from breeding catteries where an outbreak of respiratory disease was occurring. Thus, many of the FHV-1 isolated cats came from the same location; furthermore, most of the vaccinated cats used in this study came from the same cattery.

In addition, FCV was the virus most frequently isolated from cats that did not show signs of disease (Table 2). As previously noted, FHV-1 is typically isolated when animals present clinic manifestations, whereas FCV is isolated when they do not (12). Again, virus biology is the most probable explanation for these results because FCV carriers excrete the virus continuously, whereas FHV-1 carriers excrete the virus only when it is reactivated (11, 13, 25). The percentages of isolation were roughly 60% for FCV and 27% for FHV-1 (Table 2). A comparison with other data shows only that FCV is more commonly isolated than FHV-1 because the prevalence detected varied as much as 15 to 25% for FCV and 0.2 to 33.3% for FHV-1 in one study (12) to 25% for FCV and 0.6% for FHV-1 in another study (3).

However, no virus was isolated from approximately 64% of the samples coming from cats showing signs of respiratory disease (Table 1). This finding could be attributed to problems with sampling and storage conditions or even cases in which cats were sampled late in the course of disease, as has been reported in other studies (2, 29). Nonetheless, there are other causes for respiratory and ocular diseases in felines other than FCV and FHV-1, including agents such as fungi, bacteria and other viruses (10). *Chlamydophila felis* is a bacteria routinely identified in cases of conjunctival disease in cats. *Bordetella* spp., which is associated with mild respiratory signs, is also commonly identified (2). Thus, cases of disease in cats from which neither virus was isolated could have other etiologic causes.

The age of the individuals was taken in to account and when the results were analyzed

separately for each virus, it was observed that FCV was primarily isolated from adult cats, whereas FHV-1 was primarily isolated from younger animals (results shown in Table 2). A higher prevalence of FCV in adults has been already described (34). An average age of 38 months for cats was demonstrated positive for FCV, and 29.9 months for FHV-1-positive cats (34). The results for FHV-1 also match those of previous studies, which isolated FHV-1 from 16.9% of cats between one and three months old, 8.7% from cats between 4 and 11 months old, and less than half the percentage from cats above 11 months of age (2).

As previously noted, the female/male ratio was comparable, whereas the viral isolation frequency was twice more frequent in females than males (Table 1 and Results). The large amount of positive samples obtained from females was not an expected result. In quite a few studies performed in cat populations from numerous locations, no gender difference was observed (11, 25).

The castration status of the cats appears to play a larger role than the gender in the epidemiology because some researchers have shown a smaller number of positive spayed females and neutered males in comparison to non-castrated cats (2, 30). The higher prevalence of the virus among non-castrated cats has been attributed to the behavior of these animals; the likelihood of exposure to virus of neutered/spayed animals may be reduced because social interactions are less likely to occur (30). In the present study, it was not possible to compare the virus distribution among castrated cats because the data were not available.

The viruses were more often isolated from cats that shared the same habitat (Table 1). Such results are similar to those demonstrated in surveys performed in European countries (2, 13). The high prevalence of these viruses in cats that share a living space is generally attributed to the method of transmission, which requires close contact between infected and susceptible animals (11, 25). Furthermore, another important characteristic of the Brazilian cat population is

that most cats, even ones with owners, live outside. This means that cats are more exposed to infectious diseases that are transmitted by contact than the general population analyzed in surveys performed in the USA and European countries.

Taken together, the data shown here provide insight into the epidemiology of FCV and FHV-1 among the cat population in counties in southern Brazil. One point of particular concern is the detection of cats without clinical signs that excrete the viruses. Critically, this occurred even among animals that were vaccinated. Such animals could be a source of infection, particularly for kittens. In addition, because FCV is an RNA virus that varies widely, the molecular characterization of isolates of this virus is underway in our lab, and the data compiled in this article will aid in further studies relating to epidemiology and vaccine efficacy.

Both FCV and FHV are prevalent in Southern Brazil despite sporadic vaccination. Cats likely encounter these viruses at a very young age and this contributes to a complicated epidemiology for both these viruses. Of significance cats showing no clinical signs were found to be excreting virus as well as those that had previously been vaccinated against FCV and FHV. This highlights the need for continued research into these important diseases of domesticated cats.

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Table 1. General description of the feline population and the distribution of virus isolation among the samples collected from domestic cats from July 2006 to June 2009.

Epidemiologic aspect	Overall (%)	Virus isolation (%)	
		Positive	Negative
Total of cats	302	55 (18.2)	247 (81.8)
Age (years)			
< 1 year	116 (38.4)	24 (20.7)	92 (79.3)
1 - 5 years	144 (47.7)	27 (18.8)	117 (81.2)
5 - 10 years	34 (11.2)	4 (11.8)	30 (88.2)
> 10 years	8 (2.6)	0 (0)	8 (100)
Gender			
female	146 (48.3)	37 (25.3)	109 (74.7)
male	142 (47)	14 (9.9)	128 (90.1)
not informed	14 (4.6)	4 (28.6)	10 (71.4)
Vaccination status			
vaccinated	89 (29.5)	14 (15.7)	75 (84.3)
not vaccinated	186 (61.6)	36 (19.3)	150 (80.7)
not informed	27 (8.9)	5 (18.5)	22 (81.5)
Habitat			
single	27 (8.9)	2 (7.4)	25 (92.6)
with other cats	253 (83.7)	52 (20.6)	201 (79.4)
not informed	22 (7.2)	1 (4.5)	21 (95.5)
Clinical signs			
presence	70 (23.2)	25 (35.7)	45 (64.3)
absence	232 (76.8)	30 (12.9)	202 (87.1)
Swabs collected	572	73 (12.8)	499 (87.2)
conjunctival	289 (50.5)	32 (11.1)	257 (88.9)
nasal	280 (49)	38 (13.6)	242 (86.4)
oral	2 (0.3)	2 (100)	0 (0)
oropharyngeal	1 (0.2)	1 (100)	0 (0)

Table 2. Characteristics of the 55 positive cats in terms of isolation of feline calicivirus (FCV), feline herpesvirus type 1 (FHV-1) or both viruses.

Epidemiologic aspect	Virus isolation (%)			
	FCV	FHV-1	Both	Overall
Total	29 (52.7)	21 (38.2)	5 (9.1)	55
Age (years)				
< 1 year	11 (45.8)	9 (37.5)	4 (16.7)	24 (43.6)
1 - 5 years	16 (59.3)	10 (37)	1 (3.7)	27 (49.1)
5 - 10 years	2 (50)	2 (50)	- (0)	4 (7.3)
Gender				
female	21 (56.8)	14 (37.8)	2 (5.4)	37 (67.3)
male	8 (57.1)	6 (42.9)	- (0)	14 (25.4)
not informed	- (0)	1 (25)	3 (75)	4 (7.3)
Vaccination status				
vaccinated	3 (21.4)	10 (71.4)	1 (7.1)	14 (25.4)
not vaccinated	23 (63.9)	10 (27.8)	3 (8.3)	36 (65.4)
not informed	3 (60)	1 (20)	1 (20)	5 (9.1)
Habitat				
single	2 (100)	- (0)	- (0)	2 (3.6)
with other cats	27 (51.9)	21 (40.4)	4 (7.7)	52 (94.5)
not informed	- (0)	- (0)	1 (100)	1 (1.8)
Clinical signs				
presence	11 (44)	13 (52)	1 (4)	25 (45.5)
absence	18 (60)	8 (26.7)	4 (13.3)	30 (54.5)

3. CAPÍTULO 2

Genetic and phylogenetic analyses of capsid protein gene in feline calicivirus isolates from Rio Grande do Sul in southern Brazil

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Abstract

Feline calicivirus (FCV) is an important pathogen that affects domestic cats, inducing acute oral and upper respiratory tract clinical signs. The aim of this study was to analyze the variability of the capsid protein in different FCV isolates from southern Brazil. The sequencing analyses of thirteen Brazilian FCV samples, phylogenetic analyses and assessments of ten previously published sequences were conducted by examining the open reading frame 2 (ORF2, regions B-F). Comparisons of the predicted amino acid sequences of the ORF2 in Brazilian FCV isolates with those of the FCV-F9 strain indicated that the main differences are located within the regions C and hypervariable E (HVR_E). Epitopes that were mapped to the regions D, 5'HVR_E and conserved E also presented with some variability when compared to the strain F9. This is the first study describing sequence analyses and the phylogenetic relationships among FCV isolates from Brazil. The results presented here may expand upon current knowledge regarding aspects of FCV biology, epidemiology and genetic diversity and provide insights into improving the efficacies of current FCV vaccines.

Keywords: FCV, ORF2, Brazilian isolates, Molecular diversity

The feline calicivirus (FCV) is a highly infectious pathogen that affects cats worldwide (Radford et al., 2007). This virus is associated with a variety of clinical presentations, including respiratory disease, acute and chronic stomatitis, acute arthritis, limping syndrome and hemorrhagic-like fever, also known as FCV-associated virulent systemic disease (FCV-VSD) (Pedersen et al., 2000; Radford et al., 2007). FCV is a member of the genus *Vesivirus* in the family *Caliciviridae*. The FCV genome consists of a positive-sense, single-stranded RNA molecule of approximately 7.7 kb that contains three open reading frames (ORF) (Radford et al.,

2007). ORF1 encodes the non-structural proteins, ORF2 encodes the major capsid protein, VP1 (viral protein) and ORF 3 encodes a minor structural protein, VP2 (Neill, 1990; Neill et al., 1991; Sosnovtsev and Green, 2000).

ORF2 contains conserved and variable sequences and is divided into six regions (A-F) (Neil, 1992; Seal et al., 1993). In contrast to regions C and E, the regions B, D and F are relatively conserved among the FCV isolates (Carter et al., 1992). Region E is further divided into two hypervariable regions (5' and 3' hypervariable or 5'HVR_E and 3'HVR_E, respectively) that are separated by a conserved central sequence, named conserved region E (conE) (Seal et al., 1993; Seal, 1994). The variable region E contains the major B-cell epitopes, making this region a target for virus-neutralizing antibodies (Tohya et al., 1997; Radford et al., 1999; Geissler et al., 2002).

The basis of FCV antigenicity resides in the capsid protein, which is important for receptor binding, internalization and uncoating (Geissler et al., 2002). Additionally, the RNA genome of FCV has elevated mutation rates that contribute to its molecular diversity, and it is therefore referred to as a quasispecies (Kreutz et al., 1998; Radford et al., 1998). Vaccinations for FCV were initiated in the 1970s, and the majority of vaccines available worldwide are based on the broadly cross-reactive FCV-F9 strain, although other FCV strains are commercially used as well, such as F7 and 255 (Radford et al., 2006). Despite the use of vaccines, FCV remains widespread in the domestic cat population (Radford et al., 2007).

In Brazil, little is known about FCV in the domestic cat population. After its first isolation (Weiblen et al., 1988), the pathogenicity was analyzed (Pereira et al., 1994), and some serological studies were also conducted in non-domestic (Filoni et al., 2006) and domestic felines (Johann et al., 2009). A study performed by our group between the years of 2006-2009, reported the isolation of FCV and the feline herpesvirus type-1 in domestic cats from southern Brazil

(author's data). The aim of the present study was to investigate the variability in the capsid proteins of different Brazilian FCV isolates and compare them to the reference FCV-F9 strain and other published FCV sequences. This is the first report of sequence and phylogenetic analyses of FCV Brazilian isolates.

The origin, clinical history and GenBank access number of the thirteen FCV Brazilian isolates and the ten previously published sequences that were analyzed in this study are listed in the table 1. The CRFK (*Crandell-Reese feline kidney*) cells were used for virus isolation and amplification. The RNA extraction was performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNA samples were reverse transcribed (SuperScriptTM III RT – Invitrogen). The ORF2 (regions B-F) of the FCV isolates was PCR amplified. The primers used for the PCR were designed based on the ORF2 sequence of the complete genome of the FCV-F9 strain and included FCV_Capfor 5'–TTCGGCCGTTTGTCTTCC–3' [position 6401-6419 (region B of ORF2)] and FCV_Caprev 5'–TTGTGAATTAAAGACATCAATAGACCT–3' [position 7080-7053 (region F of ORF2)], resulting in a 679 bp product (Fig. 1). The commercial live attenuated vaccine, Felocell CVR-C (Pfizer Animal Health, USA), was used to optimize the PCR reaction and as a positive control in all reactions. The negative control consisted of mock-infected CRFK cells. The PCR products were purified using the PureLink PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purified products were sequenced in triplicate in a MEGABACE sequencer (Amersham Biosciences). Each isolate was sequenced at least nine times (forward and reverse directions) to prevent the misreading of the cDNA sequences. The same PCR primers were used for the sequencing.

The cDNA sequencing data were analyzed using the EditView 1.0.1, Genejockey and SeqEdv1.0.3 software packages. The nucleotide analyses of the cDNAs, deduced amino acid sequences, alignments, phylogenetic analyses and distance value of the nucleotide sequences

from region E for the Brazilian isolates and the ten previously published strains of FCV were conducted using the molecular evolutionary genetics analysis (MEGA 4.0) (Tamura et al., 2007). One thousand bootstrap repetitions were performed for each analysis. The evolutionary history used to construct the phylogenetic tree was performed using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Linus, 1965). Positions that contained gaps and/or missing data were eliminated.

The amino acid sequence alignment of the thirteen Brazilian isolates is shown in Fig. 2. The capsid protein of FCV is an important target for the immune response, and serologic and molecular studies have mapped linear epitopes to the ORF2 sequence (Radford et al., 1999; Geissler et al., 2002). These epitopes are located in the regions D, 5'HVR_E and conE, which are indicated by black circles in Fig. 2. The alignment showed that all the thirteen isolates possessed the same conserved amino acid sequence in the region D. Additionally, SV1425/93 and SV160/02 showed the same amino acid sequence for the epitopes of the region 5'HVR_E. On the other hand, high diversity was observed in the region 5'HVR_E of all other Brazilian isolates (Fig. 2). SV520/09 had the highest amino acid divergence with a difference of eight amino acids compared to the F9 strain, while the remaining ten isolates showed divergences for four or five amino acids (Fig. 2). Besides, the amino acid sequence NGT (Asn-Gly-Thr) at positions 439-441 of the region 5'HVR_E (underlined region in Fig. 2) has been reported to have strong reactivity to antibodies (Seal, 1994). Increased amino acid variability was observed in this region for the Brazilian isolates (Fig. 2).

FCV has only one serotype, but the FCV strains isolated in different regions and periods apart in the same population show a great diversity (Geissler et al., 1997; Glenn et al., 1999; Sato et al., 2002). Cross-neutralization studies have demonstrated that hyperimune serum raised

against the F9 strain may not neutralize field isolates *in vitro* (Povey, 1974; Knowles et al., 1990). For instance, the hyperimmune serum raised against F9 was not cross-protective for the Australian isolates of FCV (Baulch-Brown et al., 1999) as well German isolates (Geissler et al., 1997). The lack of cross-neutralization among FCV strains has been related to the variation in the sequences of the antigenic region of FCV (Radford et al., 1999).

The quasispecies generation observed in the FCV and other RNA viruses are attributed to the low fidelity of the RNA polymerase along the viral replication (Domingo et al., 1997). The presence of quasispecies for FCV has been detected *in vitro* and *in vivo*, although the highest substitution rates were observed *in vivo*; probably due to the host factors like the immune response (Radford et al., 1998). The genetic diversity has been considered as a mechanism for FCV to evade from the immune system in the persistently infected cat (Kreutz et al., 1998; Radford et al., 1998). Thus, the observed differences in this region may potentially interfere with vaccine protection conferred to the cats in Brazil since the results of the current study suggest that vaccine strains used in Brazil are the same as worldwide.

The feline junctional adhesion molecule 1 (fJAM-1), a protein of the immunoglobulin superfamily, was found to be the FCV functional receptor in cells (Makino et al., 2006); and the X-ray structure of the FCV bound to its receptor was described (Bhella et al., 2008). The VP1 of the caliciviruses is divided in two domains; S and P, according to the X-ray structure. The P domain protrudes from the capsid surface and is divided in P1 and P2 subdomains. The neutralizing epitopes described for FCV are mapped to the P2, which is the most exposed subdomain (Chen et al., 2006; Bhella et al., 2008). Mutations in some residues of the P2 region of FCV produced viruses unable to bind to fJAM-1 soluble receptor, although these mutants were able to bind to fJAM-1 expressing cells (Ossiboff et al., 2010). In the FCV Brazilian isolates, only a few mutations were detected on the P2 subdomain in the same position as described by

Ossiboff et al. (2010) (Fig.2). It was suggested that the region comprehending the 5'HVR_E could tolerate significant sequence alterations without interfering with the folding of this subdomain (Chen et al., 2006). If we take in consideration the suggestion of Chen et al. (2006) maybe, the biological significance of the mutations present on the FCV Brazilian isolates has to be further evaluated.

Phylogenetic tree was constructed using the deduced amino acid sequences from the thirteen Brazilian isolates and ten published FCV sequences (Fig. 3). The sequence of the San Miguel sea lion virus type 1 (SMSV-1) [GenBank ID: ACC57040] was used as an outgroup (see Fig. 3). The Brazilian FCV isolates showed similar distributions in the phylogenetic tree as has been described for FCV strains/isolates from different regions (Geissler et al., 1997; Glenn et al., 1999).

The homology among the isolates SV160/02 and SV1425/93 and the standard F9 strain may indicate a close relationship. SV1425/93 and SV160/02 were isolated in 1993 and 2002, respectively. The distances that were observed for the nucleotides of region E (data not shown) and the phylogenetic tree among these viruses and the other Brazilian isolates from 2006 to 2009 suggest that the FCV isolates are evolving. Another possible indication of evolution is the detection of an insertion of three amino acids (DNN) into the region 3'HVR_E of the capsid protein in the two isolates that is also present in the F9 standard strain, although it is absent in the other eleven Brazilian isolates (Fig. 2). In addition, the FCV that were isolated from different locations did not possess the DNN sequence (Geissler et al., 1997; Glenn et al., 1999).

Most of the FCV from the Brazilian cats, clustered into two major groups (Fig. 3). The presence of a genogroup among FCV strains was demonstrated in a survey that was conducted in Japan (Sato et al., 2002), which, however, included a greater number of samples than the present study. Based on the results shown here, it cannot be assumed that the FCV isolates from Brazil

would form genogroups. Although this tendency has been observed in Japanese strains (Sato et al., 2002), none of the other FCV viruses that were isolated from different geographic locations showed a tendency to form genogroups (Glenn et al., 1999).

In conclusion, the FCV problem in Brazil is similar to that in other places of the world where the epidemiology and molecular biology of the virus have been studied. The sequencing of the variable regions of the FCV capsid from the thirteen Brazilian isolates showed diversity when compared to the F9 vaccine strain and to each other. The sequence diversity mapped mainly to the regions for which neutralizing epitopes were described, suggesting possible interference with vaccine protection. Further studies on antigenicity among the isolates may highlight this issue.

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Table 1 – Brazilian isolates and previously published strains of feline calicivirus (FCV).

FCV isolate/strain (# animal)	Year/country	Clinical origin	Age/gender	Vaccination status	Samples (swab)	GenBank access number
SV520/09#2	2009/Brazil[a]	Stomatitis in the faucets	12 years/male	No	Oral	HQ260661
SV147/09	2009/Brazil	Acute respiratory disease	4 months/female	Yes	Nasal	HQ260673
SV45/09	2009/Brazil	Without	1 year/female	Yes	Nasal	HQ260672
SV306/08	2008/Brazil	Oral disease, ulcers in both lips	1 year/male	Yes	Oral	HQ260671
SV57/08	2008/Brazil	Without	adult/female	No	Conjunctival and nasal	HQ260670
SV56/08	2008/Brazil	Without	3 months/female	Ni[b]	Nasal	HQ260669
SV55/08	2008/Brazil	Without	1.5 years/male	No	Conjunctival	HQ260668
SV38/08	2008/Brazil	Acute respiratory signs and with 7 months old had pneumonia	8 years/female	No	Conjunctival	HQ260667
SV142/07#07	2007/Brazil	Acute respiratory disease	2 months/female	No	Conjunctival and nasal	HQ260666
SV127/07	2007/Brazil	Without	adult/female	Ni	Nasal	HQ260665
SV368/06#11	2006/Brazil	Acute respiratory disease	2 months/female	No	Conjunctival and nasal	HQ260664
SV160/02	2002/Brazil	Ni	Ni	Ni	Conjunctival and nasal	HQ260662
SV1425/93	1993/Brazil	Ni	Ni	Ni	Ni	HQ260663
F9	1958/USA	Acute respiratory and oral disease				M86379
F4	1971/Japan	Acute respiratory signs				D90357
CFI/68	1960/USA	Acute respiratory signs and stiffness				U13992
255	1970/USA	Pneumonia and oral lesions				U07130
LLK	1983/Canada	Lameness				U07131
NADC	1993/USA	Acute respiratory signs				L09718
Urbana	1960/USA	Acute respiratory signs				L40021
KCD	1957/New Zealand	Acute respiratory signs				L09719
2280	1982/Canada	Limping syndrome				X99445
F65	1990/UK	Lameness and oral lesion				AF109465

[a] Isolate obtained from Porto Alegre, all the others were isolated from Santa Maria/Rio Grande do Sul/Brazil

[b] Not informed

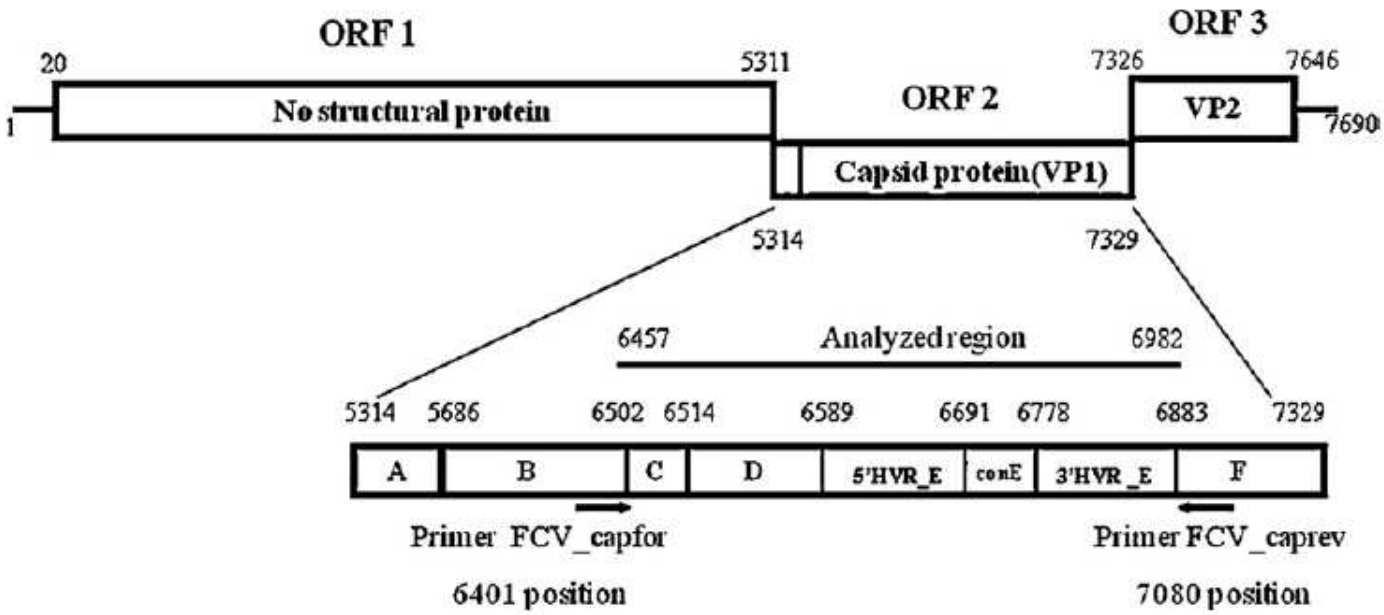

Fig.1. The genome map of the feline calicivirus (FCV), showing the sequence amplified by the primers set and the region of open reading frame 2 (ORF2) analyzed in this study.

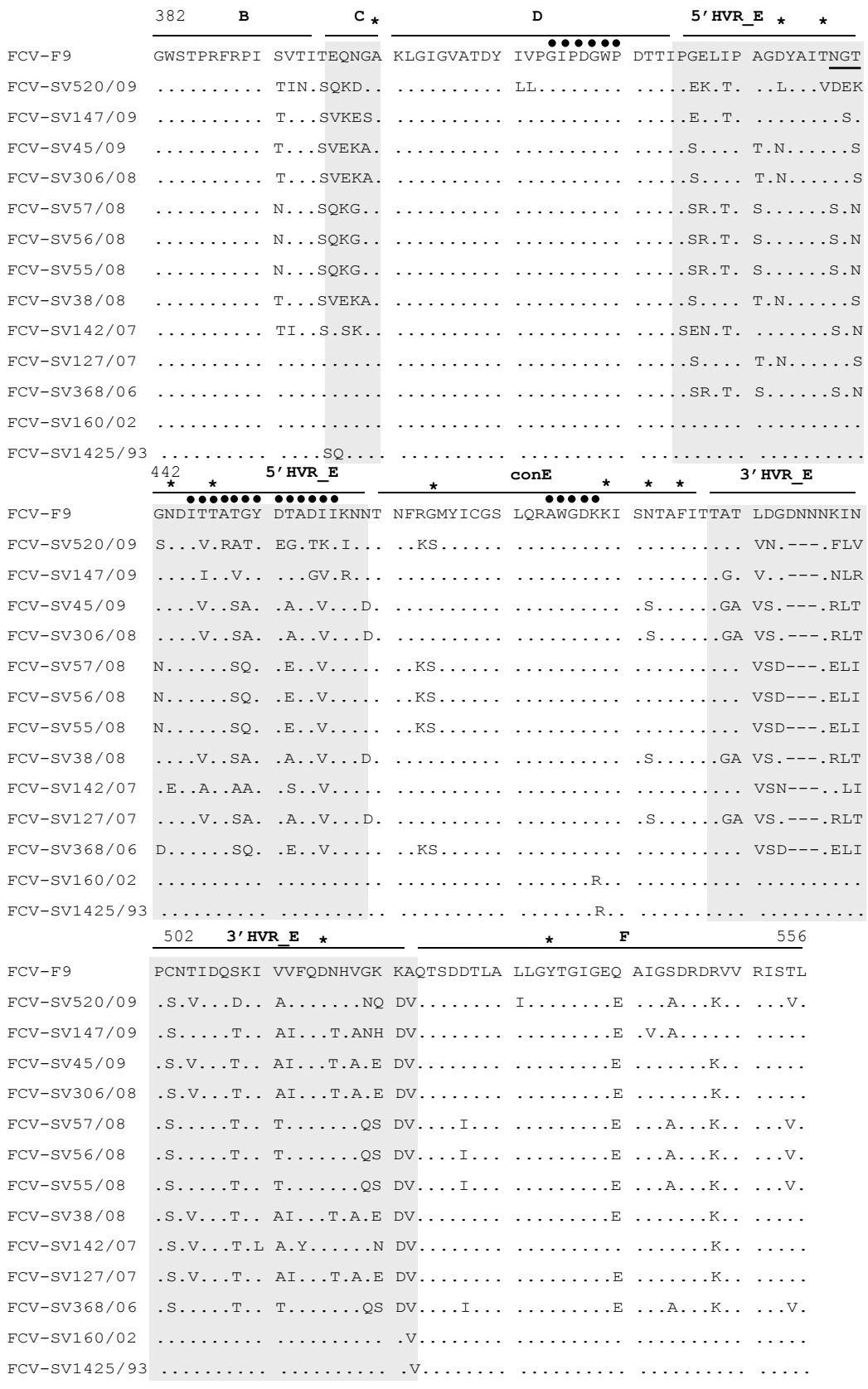
382 B C D 5′HVR_E
FCV-F9 GWSTPRFRPI SVTITEQNGA KLGIGVATDY IVPGIPDGWP DTTIPGELIP AGDYAITNGT
FCV-SV520/09 ..... TIN.SQKD.. ..... LL..... .....EK.T. ..L...VDEK
FCV-SV147/09 ..... T...SVKES. ....E..T. .....S.
FCV-SV45/09 ..... T...SVEKA. ....S.... T.N.....S
FCV-SV306/08 ..... T...SVEKA. ....S.... T.N.....S
FCV-SV57/08 ..... N...SQKG.. ....SR.T. S.....S.N
FCV-SV56/08 ..... N...SQKG.. ....SR.T. S.....S.N
FCV-SV55/08 ..... N...SQKG.. ....SR.T. S.....S.N
FCV-SV38/08 ..... T...SVEKA. ....S.... T.N.....S
FCV-SV142/07 ..... TI..S.SK.. ....SEN.T. .....S.N
FCV-SV127/07 ....S.... T.N.....S
FCV-SV368/06 ....SR.T. S.....S.N
FCV-SV160/02 ....
FCV-SV1425/93 ....SQ.... ....
442 5′HVR_E conE 3′HVR_E
FCV-F9 GNDITTATGY DTADIIKNNT NFRGMYICGS LQRAWGDKKI SNTAFITTAT LDGDNNNKIN
FCV-SV520/09 S...V.RAT. EG.TK.I... ..KS..... .... VN.---.FLV
FCV-SV147/09 ....I..V.. ...GV.R... ....G. V..---.NLR
FCV-SV45/09 ....V..SA. .A..V...D. .... .S.....GA VS.---.RLT
FCV-SV306/08 ....V..SA. .A..V...D. .... .S.....GA VS.---.RLT
FCV-SV57/08 N.....SQ. .E..V..... ..KS..... .... VSD---.ELI
FCV-SV56/08 N.....SQ. .E..V..... ..KS..... .... VSD---.ELI
FCV-SV55/08 N.....SQ. .E..V..... ..KS..... .... VSD---.ELI
FCV-SV38/08 ....V..SA. .A..V...D. .... .S.....GA VS.---.RLT
FCV-SV142/07 .E..A..AA. .S..V..... .... VSN---..LI
FCV-SV127/07 ....V..SA. .A..V...D. .... .S.....GA VS.---.RLT
FCV-SV368/06 D.....SQ. .E..V..... ..KS..... .... VSD---.ELI
FCV-SV160/02 ....R.. ....
FCV-SV1425/93 ....R.. ....
502 3′HVR_E F 556
FCV-F9 PCNTIDQSKI VVFQDNHVGK KAQTSDDTLA LLGYTGIGEQ AIGSDRDRVV RISTL
FCV-SV520/09 .S.V...D.. A.....NQ DV..... I.....E ...A...K.. ...V.
FCV-SV147/09 .S.....T.. AI...T.ANH DV..... .....E .V.A..... .....
FCV-SV45/09 .S.V...T.. AI...T.A.E DV..... .....E .....K.. .....
FCV-SV306/08 .S.V...T.. AI...T.A.E DV..... .....E .....K.. .....
FCV-SV57/08 .S.....T.. T.....QS DV....I... .....E ...A...K.. ...V.
FCV-SV56/08 .S.....T.. T.....QS DV....I... .....E ...A...K.. ...V.
FCV-SV55/08 .S.....T.. T.....QS DV....I... .....E ...A...K.. ...V.
FCV-SV38/08 .S.V...T.. AI...T.A.E DV..... .....E .....K.. .....
FCV-SV142/07 .S.V...T.L A.Y.....N DV..... ....K.. .....
FCV-SV127/07 .S.V...T.. AI...T.A.E DV..... .....E .....K.. .....
FCV-SV368/06 .S.....T.. T.....QS DV....I... .....E ...A...K.. ...V.
FCV-SV160/02 .... .V..... ....
FCV-SV1425/93 .... .V..... ....

Figure 2

Fig. 2. Alignment of the amino acid sequences of the thirteen Brazilian feline calicivirus (FCV) isolates compared to the sequence of FCV-F9 strain. The ORF2 regions (B-F) of capsid protein gene, as proposed by Seal et al. (1993), are indicated by bars above the alignment. The first amino acid is equivalent to position 382 of the FCV capsid protein (region B, ORF2), and the last position is the amino acid 556 (region F, ORF2). In this figure, region B ranges from amino acid 382 to amino acid 396; region C (397 – 401); region D (402 – 425); region E (426 – 524) and region F from amino acid 525 to amino acid 556. Shaded regions correspond to the variable C and hypervariable E (5'HVR_E and 3'HVR_E); and the positions of linear epitopes of the FCV-F9 strain are indicated by black balls (Radford et al., 1999). The position of amino acid 439 – 441 on the region 5'HVR_E, cited on the text, is underlined. The absence of the insertions at the region 3'HVR_E are represented by – . The equivalent positions of the amino acid residues of the FCV-F5 strain identified by Ossiboff et al. (2010), a important for soluble receptor neutralization; are represented by * from region C until region F (E401K, D434N, T438I/A, N443S, T447I, S465N, K480R/E/Q, N483S, F486L, V516I/A and I535V).

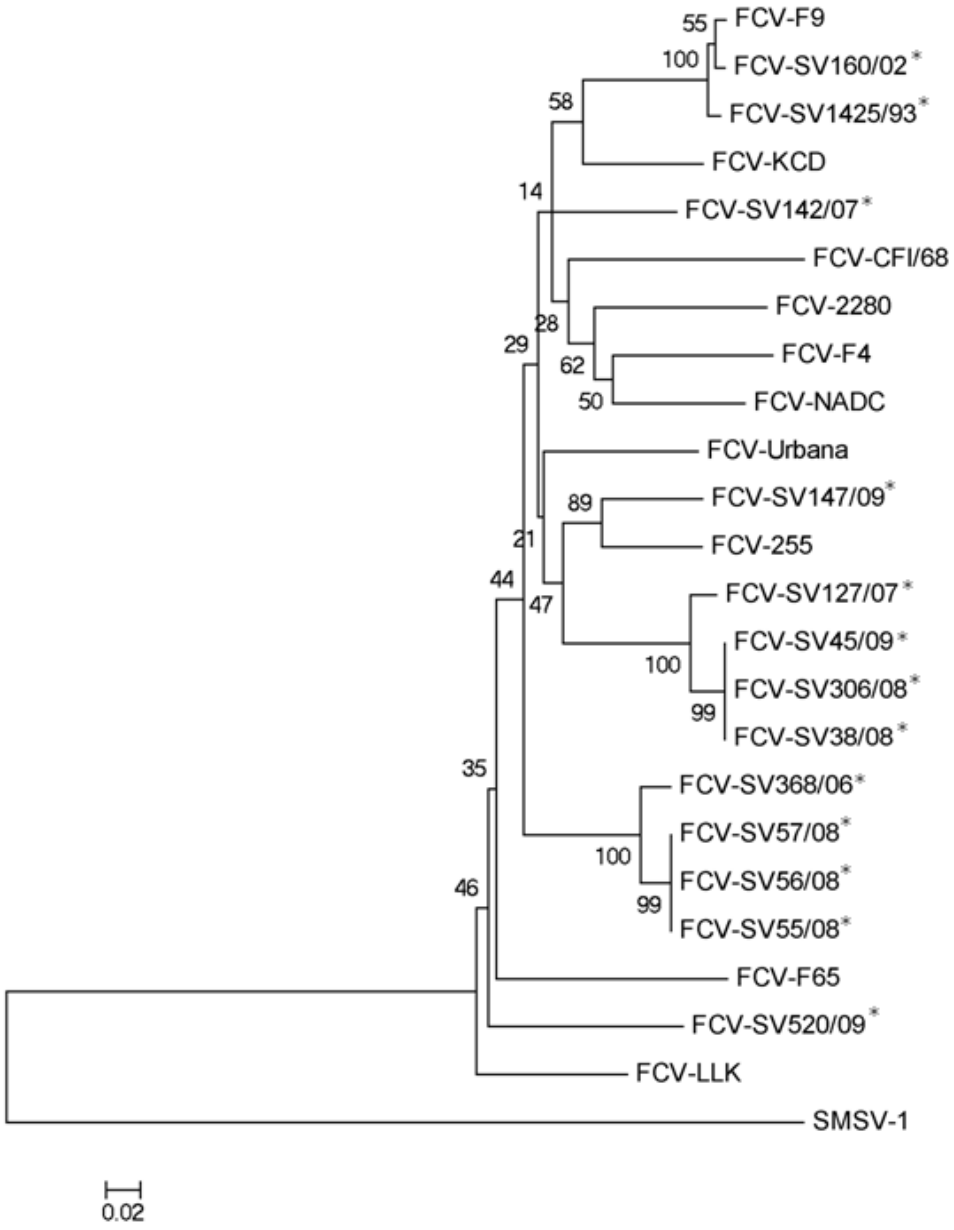

Fig. 3. Phylogeny tree of the amino acid sequence of the capsid protein gene from Brazilian feline calicivirus (FCV) isolates and previously published sequence listed in table 1 rooted with San Miguel sea lion virus type 1 (SMSV-1) as the outgroup. The analysis involved twenty-four amino acid sequence of ORF2, from region B-F, capsid protein gene. There were a total of 175 positions in the final dataset. The Brazilian FCV isolates are represented with *.

4. CAPÍTULO 3

Prevalence of feline calicivirus and herpesvirus in Rio Grande do Sul, Brazil

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ABSTRACT. – Henzel A., Brum M.C.S., Lovato L.T. & Weiblen R. 2012. [**Prevalence of feline calicivirus and herpesvirus in Rio Grande do Sul, Brazil.]** Prevalência de calicivírus e herpesvírus felino no Rio Grande do Sul, Brasil. *Pesquisa Veterinária Brasileira. 00(0):00-00.* Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. E-mail: rudi@smail.ufsm.br.

The feline calicivirus (FCV) and the felid herpesvirus type 1 (FeHV-1) are widely distributed in the feline population. These viruses are the main cause of respiratory disease in felines, and FCV is also enrolled in oral ulcers and a highly virulent systemic hemorrhagic syndrome. The aim of this study was to investigate the presence of antibodies against FCV and FeHV-1 in the population of domestic feline of some counties of the Rio Grande do Sul State (RS), by the virus neutralizing (VN) assay. A total of 630 serum samples of feline were provided from veterinary clinics, cats examined in veterinary hospitals at three universities from RS: UFRGS, UFSM and UPF, and also collected from household cats. The serum samples were classified according to the origin, gender, age and also vaccination status. The groups with the higher number of samples were male cats, 1 to 5 years old, and cats non vaccinated, with 44% (277/630), 36.3% (229/630) e 94% (592/630), respectively. Neutralizing antibodies against one or both viruses were detected in 53.6% (338/630) of the cats sampled, 23% (145/630) of the cats were seropositive only to FCV, 14.4% (91/630) only to the FeHV-1, while 16.2% (102/630) were seropositive for FCV and FeHV-1. The data presented demonstrates that both viruses are circulating in the feline population sampled, and the number of non reagent serum and no vaccinated cats indicate that there is a large parcel of the cats population which is susceptible to the infection.

INDEX TERMS: FCV, FeHV-1, URTD, domestic feline, neutralizing antibodies.

RESUMO.- O calicivírus felino (FCV) e o herpesvírus felino tipo 1 (FeHV-1) são amplamente distribuídos na população felina. O FCV e o FeHV-1 são os principais agentes da doença respiratória dos felinos, e o FCV também está envolvido em afecções orais e uma doença sistêmica hemorrágica altamente virulenta. O presente estudo teve como objetivo investigar a presença de anticorpos contra FCV e FeHV-1 na população de felinos doméstico em algumas regiões do estado do Rio Grande do Sul (RS), pelo uso da técnica de vírus neutralização (VN). Um total de 630 amostras de soro felino foram fornecidas por clínicas veterinárias, laboratórios de patologia clínica dos hospitais veterinários de três universidades do RS: UFRGS, UFSM e UPF, e também coletadas em gatos de residências. As amostras foram organizadas de acordo com a origem, sexo, idade e também histórico de vacinação. Soro proveniente de gatos machos, entre 1 e 5 anos de idade e não vacinados foram as categorias analisadas em maior número, 44% (277/630), 36,3% (229/630) e 94% (592/630) respectivamente. Das 630 amostras testadas, 53,6% (338/630) dos gatos apresentaram anticorpos neutralizantes para um ou ambos os vírus: 23% (145/630) dos gatos foram soropositivos somente contra o FCV, 14,4% (91/630) contra o FeHV-1 e em 16,2% (102/630) para FCV e FeHV-1. Os dados presentes aqui demonstram que ambos os vírus estão circulando na população amostrada, e o número de soro não reagentes e de gatos não vacinados indicam que há uma grande parcela da população de gatos susceptíveis à infecção.

TERMOS DE INDEXAÇÃO: FCV, FeHV-1, URTD, felinos domésticos, anticorpos neutralizantes.

INTRODUCTION

Feline calicivirus (FCV) and felid herpesvirus type 1 (FeHV-1) are the main viral agents of upper

respiratory tract disease (URTD) in felines (Gaskell et al. 2007, Radford et al. 2007). FCV also induces oral disease, characterized by stomatitis and ulcers; and have been described associated with a systemic hemorrhagic syndrome named virulent systemic disease associated -FCV [VSD-FCV] (Pedersen et al. 2000, Radford et al. 2007). The VSD-FCV was not reported in Brazil to this time. After the primary infection with both viruses the infected cats may become carrier for a lifelong (Gaskell et al. 2007, Radford et al. 2007). FCV is maintained in the oropharynx of infected cats and is continually shed, while the FeHV-1 produce latent infection after the first contact and virus shedding can occur after stress situations as pregnancy, lactation and transport (Wardley 1976, Povey 1979, Radford et al. 2007, Gaskell et al. 2007).

The FCV is a non enveloped RNA virus, member of the *Caliciviridae* family, and *Vesivirus* genus (Radford et al. 2007). The isolates of FCV have a great genetic diversity, but they all belong to only one serotype (Radford et al. 2007). The immune response produced after the infection is directed against epitopes mapped in the major protein of the viral capsid encoded by the open reading frame 2 (Radford et al. 1999). Following the infection with FCV, neutralizing antibodies can be detected as easily as seven days post infection (Kahn et al. 1975). The levels of such antibodies correlate well with protection against homologous challenge but not with heterologous strains (Povey & Ingersoll 1975). Although the activity of peripheral blood T lymphocytes has been demonstrated in vaccinated cats, the significance of cytotoxic T lymphocytes to FCV protection is not known (Tham & Studdert 1987).

The FeHV-1 is an enveloped DNA virus classified at the *Herpesviridae* family, *Varicellovirus* genus; and all the isolates of FeHV-1 belong just to one serotype (Gaskell et al. 2007). The immune response after the infection is formed against the glycoproteins (gp) present in the envelope of the virion, and the neutralizing antibodies are produced mainly against gD (Burgener & Maes 1988, Spatz et al. 1994). The immune response only induces protection

against the clinical signs because natural infection or vaccination does not induce sterilizing immunity against new infections (Gaskell & Povey 1979, Gaskell et al. 2007). However, different from FCV, the FeHV-1 isolates shows more homogeneity, thus the immunity induced by natural infection or vaccination provides good protection, although latency and viral shedding after reactivation are not avoided (Orr et al. 1978, Gaskell et al. 2007).

The FCV and FeHV-1 are widespread in the domestic cat population, despite the use of the vaccines (Gaskell & Knowles 1989, Binns et al. 2000). Vaccination started in the 1970s, and the vaccines are based on live attenuated or inactivated viruses (Radford et al. 2006, Thiry et al. 2009). These vaccines are considered to be safe and effective at reducing or preventing the respiratory and oral disease by FCV, although they do not prevent re-infection or carrier states (Gaskell et al. 2007, Radford et al. 2007).

The mechanisms by which cats are protected against clinical disease by FCV and FeHV-1 infection, remain unclear (Gaskell et al. 2007, Radford et al. 2007). However, antibodies are considered to be important for protection, and are routinely measured by virus neutralizing (VN) tests (Lappin et al. 2002), even though the antibody induced by vaccination or natural infection can not be differenced by VN assays. The same tests have been used to identify those strains of FCV that induce the most broadly cross-reactive immune response for use as vaccine antigens (Dawson et al. 1993, Poulet et al. 2005); and available cross-protection studies suggest that the correlation between titer and protection is reasonable (Povey & Ingersoll 1975). However, in challenge studies, some cats with no measurable neutralizing FCV or FeHV-1 antibodies were also protected against clinical disease (Lappin et al. 2002). This suggested that cell mediated and innate immune responses also play an important part in protection (Lappin et al. 2002).

The presence of the infection by both viruses is described worldwide, by isolation (Binns et al. 2000, Harbour et al. 1991) and serologic surveys in domestic (Hellard et al. 2011) and wild

feline (Daniels et al. 1999, Hofmann-Lehmann et al. 1996, Ostrowski et al. 2003). The circulation of the viruses in Brazil has already been described through some serologic surveys against FCV and/or FeHV-1 performed in populations of domestic and wild feline (Filoni et al. 2006, Ruthner-Batista et al. 2005, Johann et al. 2009). The first isolation of FCV was described in the Southern region of Brazil (Weiblen et al. 1988), and following this, a pathogenesis study was performed with experimental infection (Pereira et al. 1994). Another epidemiologic survey by our group reported the isolation for the first time of the FeHV-1 (Henzel et al. 2012a).

The goal of this study was to demonstrate the circulation of the FCV and FeHV-1 among the cat population of the central and northern regions of the Rio Grande do Sul State, Brazil, by the neutralizing antibodies detection. An extensive survey comprehending all the State or even, the region presented in this article has not yet been done. The samples came from household cats, veterinary clinics and from cats examined in veterinary hospitals at three Universities of Rio Grande do Sul. A total of 53.6% of the serum samples were positive for the presence of neutralizing antibodies against FCV, FeHV-1 or both.

MATERIAL AND METHODS

Samples source. Serum from domestic cat were analyzed for the presence of neutralizing antibodies to feline calicivirus (FCV) and felid herpesvirus type 1 (FeHV-1) by the virus neutralization (VN) assay. The serum samples were collected between the years 2007 and 2011 from the following cities from the Rio Grande do Sul State and the region surrounding these cities: Santa Maria, Passo Fundo, and Porto Alegre. The serum samples were obtained from the veterinary hospitals (VH) of the Universidade Federal de Santa Maria (UFSM), the Universidade Federal do Rio Grande do Sul (UFRGS) and from the Universidade de Passo Fundo (UPF); and

also collected from veterinary clinics and household cats from the following cities: Cachoeira do Sul, Cruz Alta, Porto Alegre, Santa Maria and Santo Ângelo. The classification by groups according to origin, gender, age, and vaccination status are detailed in Table 1. All the animal handling procedures was performed under veterinary supervision and following the recommendations of the Brazilian Committee on Animal Experimentation (COBEA, law #6.638 of May, 8th, 1979). The experiments were approved by an institutional committee on animal welfare and ethics (UFSM - approval number # 61/2009).

Cell culture and viruses. The feline kidney cell line CRFK (*Crandell-Reese feline kidney*) was used for the amplification of the viruses and for the VN assay. Cells were routinely maintained in Eagle's minimal essential medium (MEM) containing penicillin (1.6 mg.L^{-1}), streptomycin (0.4 mg.L^{-1}), amphotericin B (2.0 mg.L^{-1}) and 10% fetal calf serum. The viruses used in the assay were the FCV isolate SV65/90 and the FeHV-1 isolate SV534/00 from the Setor de Virologia from the UFSM. Both viruses were obtained from cats with signs of respiratory tract disease, and both were well characterized in a previous study by Henzel et al. (2012a).

Virus neutralization (VN) assay. The serum samples were tested against 100-200 $TCID_{50}$.mL^{-1} of both virus in the VN assay. The sera were initially inactivated at 56°C at 30 minutes before use. A serial dilution starting at 1:5 to > 1:1280 was used for FCV and 1:2 to > 1:256 for FeHV-1. The serum and virus mixture was incubated at 37ºC for 2h and then a suspension of CRFK cells was added to each well. The plates were incubated at 37ºC and 5% CO_2 for three to five days. Neutralization titers were calculated as the reciprocal of the highest serum dilution free of cytopathic effect.

RESULTS

Serum sample. A total of 630 serum samples were analyzed as described in Table 1. A greater number of samples, totalizing 323, came from Santa Maria due to the collection in the Veterinary Hospital of the UFSM; 254 samples came from UPF, 37 samples came from UFRGS, and the remaining 16 samples came from household cats from different cities of the Rio Grande do Sul State (see Table 1).

Epidemiological aspects. The serum samples that showed positive results to the presence of neutralizing antibodies to FCV and/or FeHV-1 are described in Table 1 and figure 1. In figure 1 is described in detail the number of positive samples to each one of the viruses according to gender, age and vaccination status. From the 630 samples collected, 338 (53.6%) cats were serologically positive to FCV and FeHV-1, 145 (23%) were positive only to FCV, 91 (14.4%) only to FeHV-1, and 102 (16.2%) were positive for both viruses (see Table 1).

Regarding the gender of the cats, there were a higher number of samples from male (277) than the number collected from female (250) (Table 1). However, taken together the number of samples positive to the presence of antibodies for at least one of the viruses was 85.6% (214/250) for females while 58.8% (163/277) of the samples from males were positive (see Fig. 1). The percentage of antibody detection was higher for FCV than that for FeHV-1 for either, males or females (Fig. 1).

The age of the cats collected varied from a week to 19 years old. The cats were separated in four groups according to the age, in order to organize the data (Table 1 and Fig. 1). Most of the cats examined were in the group among 1 and 5 years old (36.3%) [Table 1], yet there was a higher percentage of positive samples for cats above 5 years old for both viruses. Taking together

the number of positive samples to FCV and FeHV-1 in the figure 1, it can be observed that 82.3% (135/164) of the samples from cats over 5 years old were positive while 70.5% (194/275) of the samples that came from cats under 5 years old were positive.

Concerning to the vaccination status, only a small parcel of the population of the cats tested were vaccinated against FCV and/or FeHV-1 since 94% (592/630) of the samples came from non-vaccinated cats, while only 6% (38/630) came from vaccinated cats (Table 1). Not all the vaccinated cats showed the presence of neutralizing antibodies against the viruses. From the 38 vaccinated cats 50% (19/38) were positive for the presence of antibodies against FCV, and 42.1% (16/38) were seropositive for FeHV-1 (Fig. 1).

Neutralizing antibody titers. The neutralizing antibody titers detected against FCV and FeHV-1 in the positive cats are demonstrated in figure 2. From 5 to > 1280, 1:5 was the dilution detected with more frequency to FCV, corresponding to 22.2% (55/247) of the samples positive to the presence of neutralizing antibodies to this virus (Fig. 2A). For FeHV-1, the titer 16 was most commonly detected representing 21.2% (41/193) of the samples positive to this virus (Fig. 2B).

When analyzing separately the neutralizing antibodies titers of the serum samples from vaccinated cats, the variation was wider. For the 19 vaccinated cats positive for FCV the titer was from 5 to 320 and for 16 vaccinated cats positive for FeHV-1 the titers varied from 8 to > 256 (data not shown).

DISCUSSION

Serum of domestic cats from some counties of the Rio Grande do Sul (RS) State, Southern Brazil were examined for the presence of neutralizing antibodies against feline calicivirus (FCV) and

felid herpesvirus type 1 (FeHV-1) through the virus neutralization (VN) assay. A total of 630 samples of serum were tested, from which 53.6% (338/630) showed neutralizing antibodies for at least one of the two viruses, while 46.4% (292/630) did not have antibodies against neither of the viruses.

The frequency of detection of neutralizing antibodies against FCV was higher than against FeHV-1 (Table 1). This propensity has also been described in other serological surveys performed in Pelotas (RS), Brazil (Johann et al. 2009), as well as in France among domestic (Hellard et al. 2011) and wild feline (Filoni et al. 2006, Ruthner-Batista et al. 2005, Ostrowski et al. 2003). In addition, a higher rate of isolation of FCV in surveys including both viruses has been described by researchers from Europe (Wardley et al. 1974, Harbour et al. 1991, Binns et al. 2000) and Japan (Mochizuki et al. 2000). A similar aspect was also observed when a survey for the virus isolation was performed among the cats coming from almost the same region from the sera tested in the present study (Henzel et al. 2012a). The reasons for such difference among the prevalence rates are mostly attributed to the viruses' biology (Gaskell & Knowles 1989, Harbour et al. 1991).

The absence of the envelope in the FCV may account for its greater resistance in the environment as compared to FeHV-1, which would facilitate the transmission of the FCV to the susceptible cat (Gaskell et al. 2007, Radford et al. 2007). Another aspect of great relevance is the antigenic variability that occurs with FCV due to the fact of this being an RNA virus, in contrast to the more stable DNA genome of the FeHV-1 (Gaskell & Knowles 1989, Gaskell et al. 2007, Radford et al. 2007). Due to the virus variability, successive re-infections confer little identity among the immune response to heterologous strains (Povey & Ingersoll 1975, Tham & Studdert 1987, Radford et al. 2009).

Moreover, both viruses are able to induce persistence in the host, although there are basic

differences among the way it occurs (Gaskell & Povey 1979, Pedersen & Hawkins 1995). FeHV-1 is maintained in latency in the neural ganglia and its recurrence may or not occur periodically, while FCV is shed continually by the carrier cat (Gaskell & Wardley 1977, Gaskell et al. 1985, Reubel et al. 1993). The constant presence of the virus in the oropharynx as it happens with FCV could contribute to a more consistent immune response than the periodic contact of the immune system with the virus as in the case of FeHV-1 (Radford et al. 1998, Thiry et al. 2009).

In addition, there is evidence that the time for the rise and fall of the immune response are different for these viruses. The rising of the antibodies in the serum is slower to FeHV-1 than for FCV since the neutralizing antibodies titers against FeHV-1 can take more than 40 days to be detected (Thiry et al. 2009), while for FCV it takes no more than 7 days after infection or immunization (Kahn et al. 1975). Furthermore, antibodies against FeHV-1 tend to disappear earlier from the cat serum than the antibodies to FCV (Crandell et al. 1961, Gaskell & Wardley 1977). Then, cats already carrying the virus but without antibodies formed against the virus would not be detected.

The presence of neutralizing antibodies against FCV and FeHV-1 in the serum of most of the cats examined indicates natural infection since only 6% (38/630) of the serum came from vaccinated felines (Table 1). Conversely to what would be expected, not all the samples from vaccinated cats presented antibodies. From the 38 vaccinated cats, 50% (19/38) were seronegative for FCV and 57.9% (22/38) were seronegative to FeHV-1 (Fig. 1). The cats included in this survey were considered vaccinated when they get at least one vaccine application in their life, though the details vaccination protocols and date were unknown. The vaccine application protocols recommended includes two or three applications at 8-9 and 12 weeks (Radford et al. 2007) or 6, 9 and 12 weeks old cats (Dawson et al. 2001), followed by yearly re-vaccinations. Thus, the absence of antibodies in the serum of part of these cats could be explained

by the incorrect vaccination protocols. However, it has to be taking in consideration that the levels of antibodies against FeHV-1 may become undetectable a short time after vaccination [about three months] (Crandell et al. 1961, Lappin et al. 2002).

The figure 2 shows the antibody titers in the serum of the cats that were positive to one or both viruses, nevertheless, a correlation between antibody titers and protection has not yet been described for the viruses tested. In the case of FCV, high values of the neutralizing antibodies titers does not mean necessarily a better protection against the infection, because the immune response to this virus is well correlated to homologous viruses but not against heterologous due to the antigenic variation (Povey & Ingersoll 1975, Radford et al. 2009). In addition, the antigenic variation among FCV isolates may have contributed for the greater number of positive samples in the lower dilution. As already described before, the SV65/90 used to perform the assay was isolated 20 years ago. It could be that this isolate would induce low cross-reaction to antibodies produced against virus that are circulating now; since it was shown that the sequence of FCV from old isolates diverged more from the virus isolated recently than it diverged among virus isolated at the same period in time (Henzel et al. 2012b).

The difference of prevalence of antibodies against either virus among the age groups is demonstrated in figure 1. The information about the distribution of prevalence of antibodies among different age groups are rare for the viruses focused in this article. However, there is a general consensus for several other viruses of feline and canine that the prevalence increases with the age due to the fact that older animals could have more chance to be exposed to the viruses (Dezengrini et al. 2007, Johann et al. 2009). In our conditions, there were a slightly higher percentage of seropositive cats to FeHV-1 in the group above 5 years old when compared to cats under this age, 39% and 32% respectively (data not shown). A high percentage, 50% (27/53) of neutralizing antibodies was also detected against FCV in the group of cats over 10 years old

while a very low percentage of cats were positive under 1 year old, 26% [12/46] (Fig. 1). A very interesting feature is that the percentage of antibodies against FeHV-1 is a little higher for cats under one year old as compared to FCV, 30.4% and 26% respectively (Fig. 1). These results reinforce data from several isolation surveys where FCV is commonly isolated from adult cats while the FeHV-1 is more prevalent among cats under one year old (Zicola et al. 2009).

The neutralizing antibodies against the viruses were detected more often in female than male cats, even though the number of samples collected from males was higher (Table 1 and Fig. 1). A higher frequency of isolation in females were described for both viruses in a survey conducted by our group in some counties of Southern Brazil (Henzel et al. 2012a). To the moment, no difference among the gender has been described in the scientific literature regarding the prevalence of FCV and FeHV-1 as measured by the detection of antibodies or virus isolation (Gaskell et al. 2007, Radford et al. 2007). Further, as it has been already pointed out in previous articles, high prevalence of the viruses has been related to the castration status of the cat, not the gender (Wardley et al. 1974, Binns et al. 2000).

The present study demonstrates the distribution of FCV and FeHV-1 infection among the feline population sampled in part of the central and north regions of the Rio Grande do Sul State. It is also evident that there is a large parcel of the population that is unprotected. The neutralizing antibodies detection indicates a more extensive contact of such population with FCV than FeHV-1 and a higher prevalence of female seropositive. The application of programs of vaccination according of the recommendation among the feline population could help to change the situation.

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Table 1 - Serum samples from domestic cats analyzed for the presence of neutralizing antibodies against feline calicivirus (FCV) and felid herpesvirus type 1 (FeHV-1) regarding origin, gender, age, vaccination status; and the number of cats serologically positive to FCV, FeHV-1 or both viruses.

Origin of serum Samples	Number of samples	Gender (%)			Age (years) (%)					Vaccination status (%)		Cats serologically positive (%)			
	(%)	Female	Male	Ni[a]	< 1	1 - 5	5 - 10	> 10	Ni	Yes	No	FCV	FeHV-1	Both	Total
UFSM – VH	323 (51.2)	132	131	60	32	132	58	28	73	7	316	71	52	57	180
UPF – VH	254 (40.3)	91	121	42	7	73	45	15	114	20	234	68	28	19	115
UFRGS – VH	37 (5.8)	21	16	-	3	15	6	10	3	9	28	4	6	21	31
House and Veterinary Clinics[b]	16 (2.5)	6	9	1	4	9	2	-	1	2	14	2	5	5	12
Total	**630**	**250/630 (39.7)**	**277/630 (44)**	**103/630 (16.3)**	**46/630 (7.3)**	**229/630 (36.3)**	**111/630 (17.6)**	**53/630 (8.4)**	**191/630 (30.3)**	**38/630 (6)**	**592/630 (94)**	**145/630 (23)**	**91/630 (14.4)**	**102/630 (16.2)**	**338/630 (53.6)**

[a] Not informed;

[b] From the following cities: Cachoeira do Sul/Cruz Alta/Porto Alegre/Santa Maria/Santo Ângelo;

VH – Veterinary Hospital;

UFRGS – Universidade Federal do Rio Grande do Sul;

UFSM – Universidade Federal de Santa Maria;

UPF – Universidade de Passo Fundo.

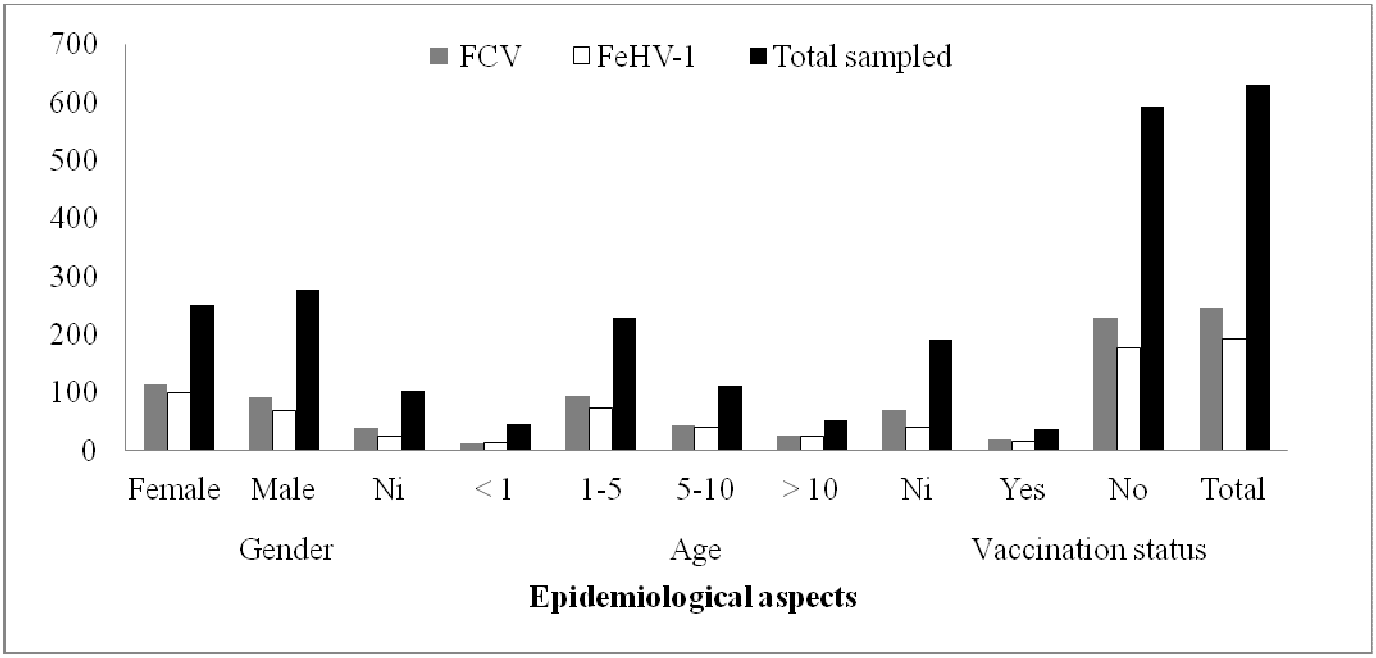
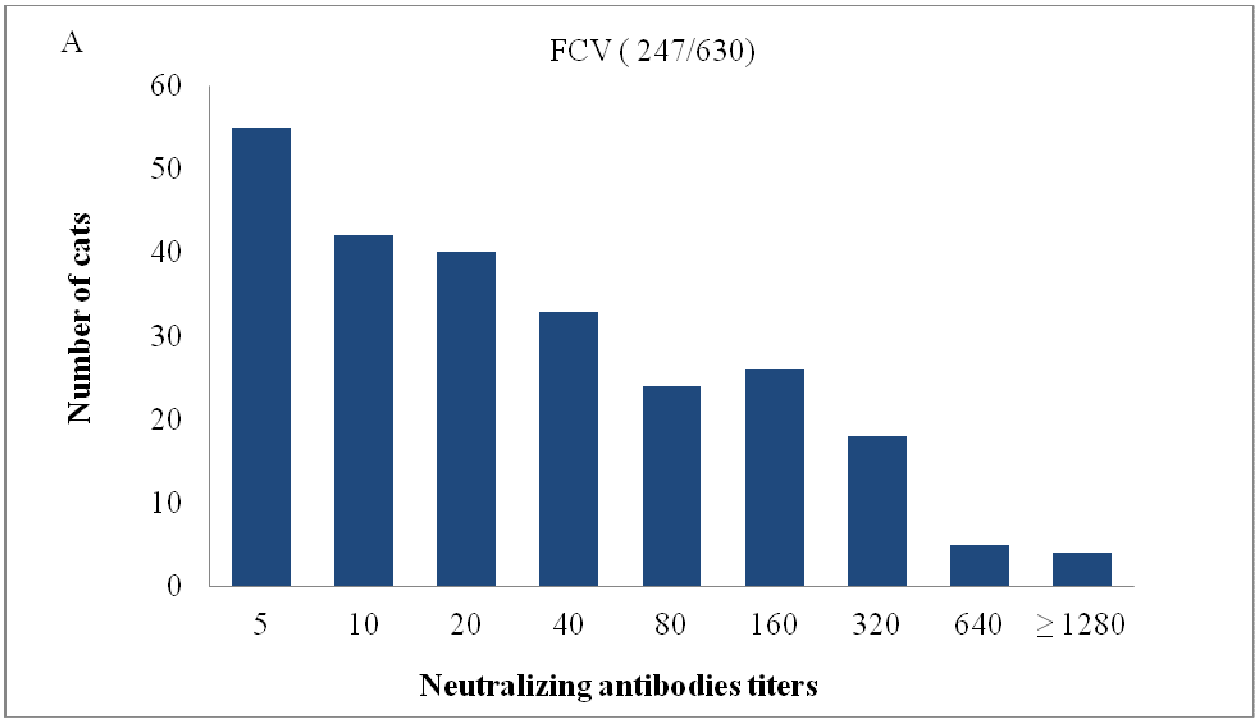


Fig. 1. Results of the number of cats positive to the presence of neutralizing antibodies against feline calicivirus (FCV) and felid herpesvirus type 1 (FeHV-1) related to the total of collected cats for each group considering gender, age and vaccination status.



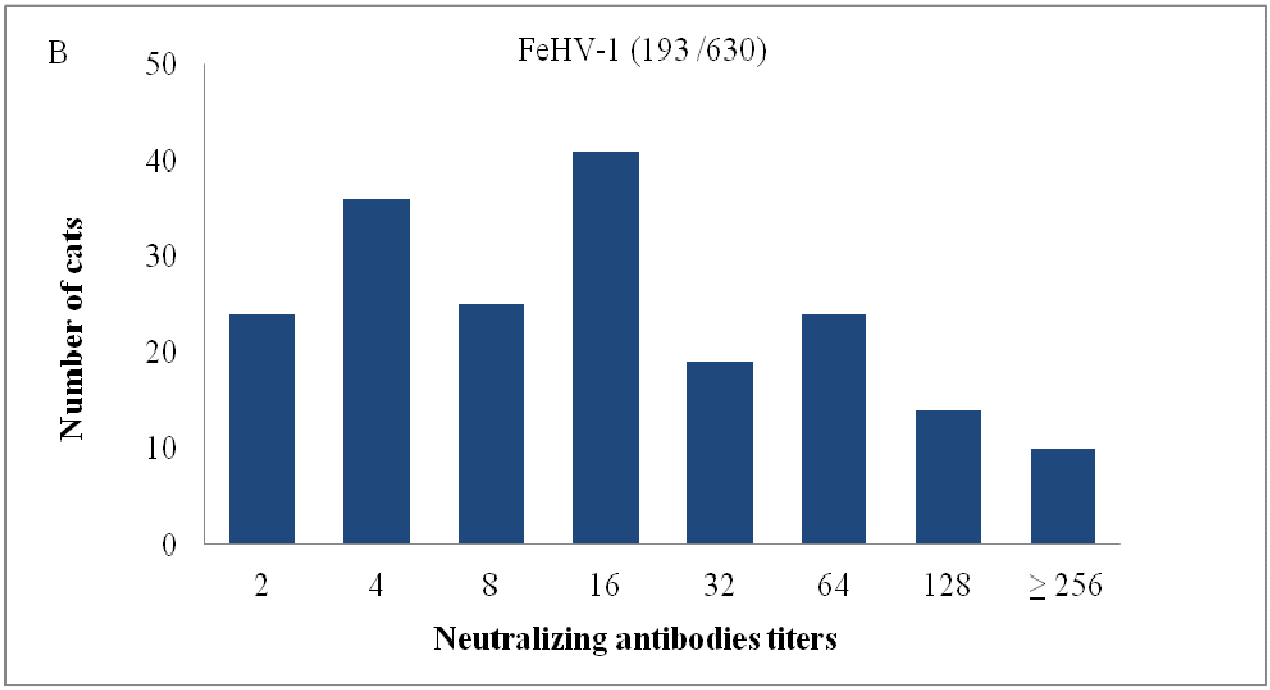


Fig. 2. Neutralizing antibodies titers detected against feline calicivirus (FCV) [A] and felid herpesvirus type 1 (FeHV-1) [B] in seropositive cats by virus neutralization assay.

5. CONCLUSÕES

Os resultados da presente tese permitem concluir que:

- O calicivírus felino (*feline calicivirus* – FCV) e o herpesvírus felino tipo 1 (*felid herpesvirus type 1* – FeHV-1) estão distribuídos na população de felinos domésticos analisados no presente estudo, Rio Grande do Sul, Brasil;

- A presença do FeHV-1 no Brasil foi confirmada por isolamento, microscopia eletrônica, imunofluorescência, PCR e sequenciamento;

- Há uma parcela da população felina reportada que é portadora do FCV e do FeHV-1;

- O FCV foi detectado com maior frequência do que o FeHV-1, tanto no isolamento quanto na sorologia;

- A presença do FeHV-1 em gatos com sinais clínicos de URTD (*Upper Respiratory Tract Disease*) foi maior do que do FCV; e a presença do FCV foi maior em gatos sem sinais clínicos;

- A região imunodominante da proteína do capsídeo do FCV (VP1), apresentou diversidade genética entre os isolados brasileiros e as cepas de referência e/ou vacinais.

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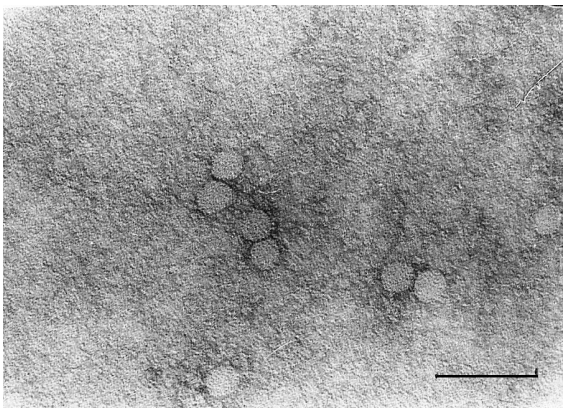
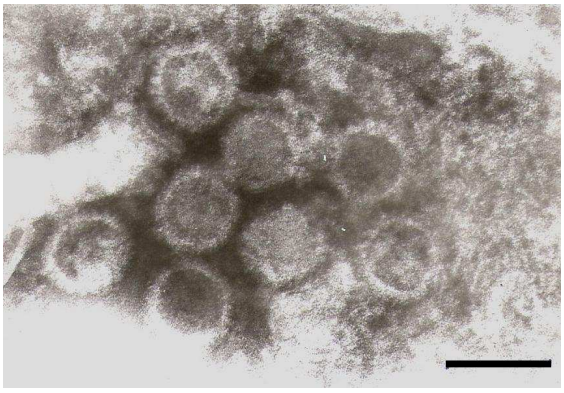
7. ANEXOS

As figuras abaixo são o resultado das técnicas de microscopia eletrônica (ME), imunofluorescência (IFA), PCR/RT-PCR (*chain reaction of polymerase/reverse transcription -chain reaction of polymerase*) e do efeito citopático em cultivo celular de origem felina (CRFK – *Crandell-Rees feline kidney*). Essas técnicas, juntamente com o sequenciamento do produto de PCR, foram utilizadas para a padronização do isolado de calicivírus felino (*feline calicivirus* – FCV) o SV65/90 e do SV534/00 isolado de herpesvírus felino type 1 (*felid herpesvirus type 1* – FeHV-1). Ambos os vírus foram definidos como vírus-padrão durante a realização da presente tese e para uso no laboratório de virologia (SV/UFSM).



A



B

FIGURA 1 - Microscopia eletrônica do isolado SV65/90 (FCV) [A] e do isolado SV534/00 (FeHV-1) [B]. A barra na figura 1A corresponde a 90nm e na figura 1B, 140nm.

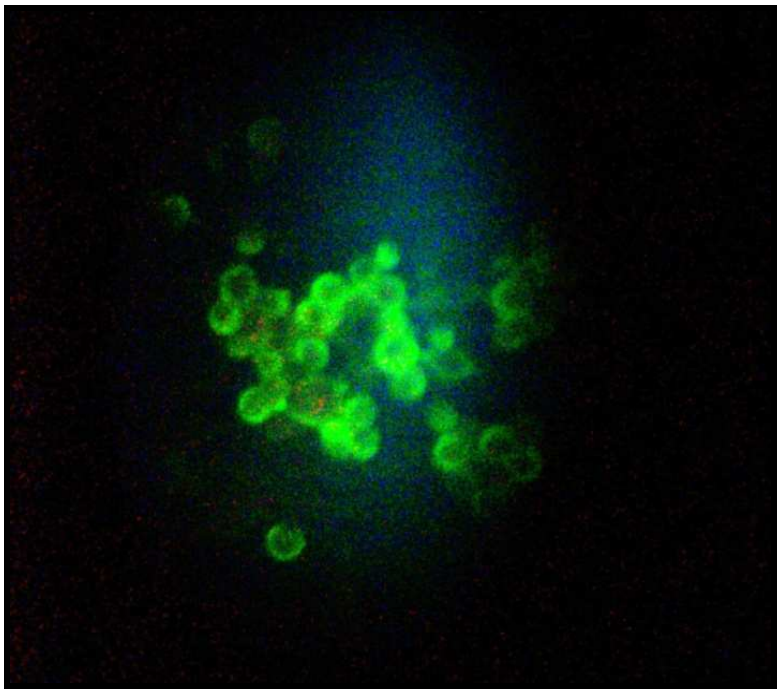


FIGURA 2 – Imunofluorescência do calicivírus felino (FCV), isolado SV65/90. O anticorpo monoclonal primário foi produzido comercialmente em camundongo contra a proteína do capsídeo do FCV [*Abcam Anti-FCV antibody - ab33990 (FCV1-43)]*. O anticorpo anti-IgG de camundongo (*Sigma*), conjugado com a enzima isoticionato de fluoresceína (FITC), foi utilizado como anticorpo secundário.

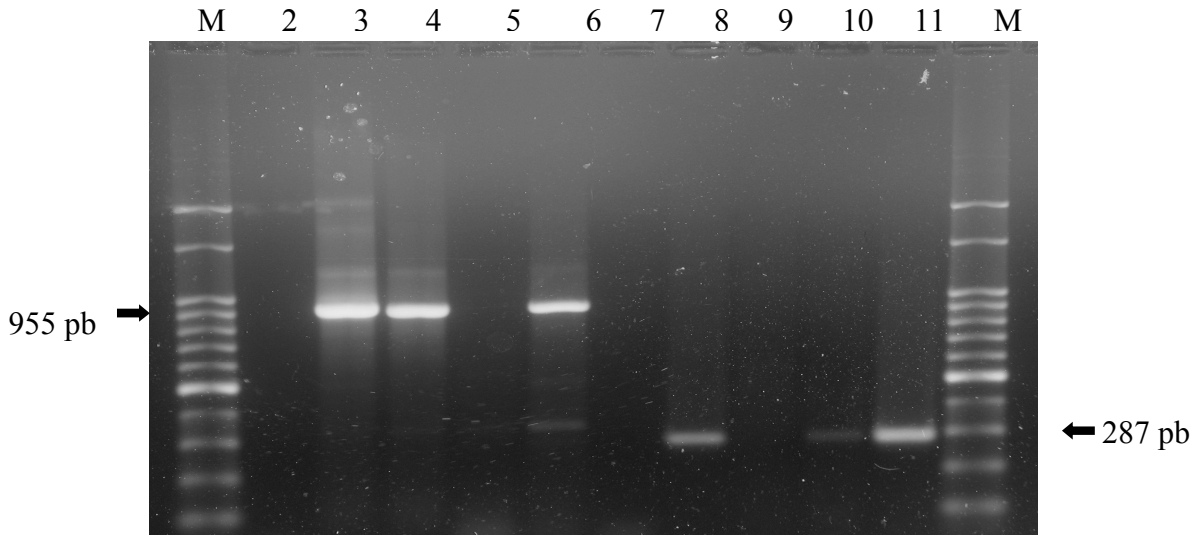
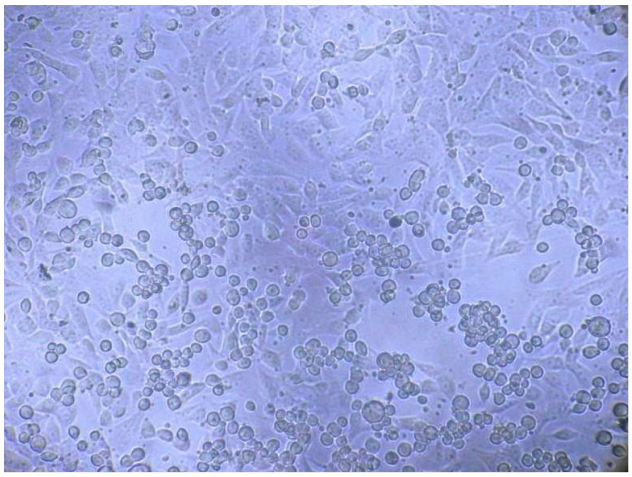
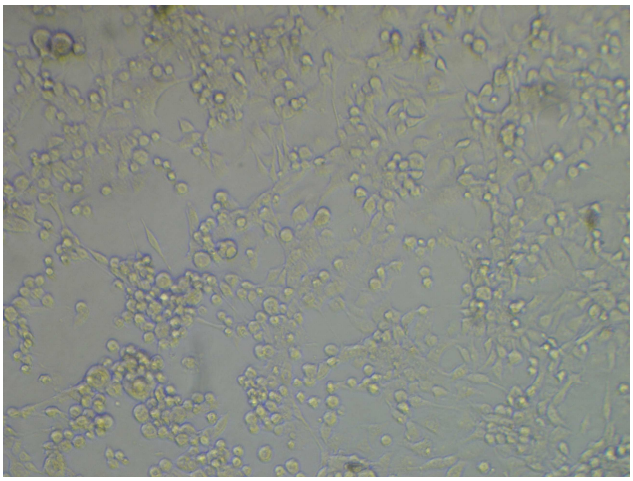


FIGURA 3 – Resultado da reação em cadeia da polimerase (PCR) para padronização do isolado de calicivírus felino (FCV) SV65/90 e do isolado de herpesvírus felino tipo 1 (FeHV-1) SV534/00. A transcrição reversa (RT-PCR) do FCV estão demonstradas entre as colunas 2 e 6, e o PCR do FeHV-1 entre as colunas 7 e 11. As colunas 1 e 12 correspondem ao marcador de peso molecular "M" (*Ludwig – Biotec*); colunas 2 e 7: controle negativo (CRFK sem inóculo); colunas 3 e 8: vacina – *Felocell*; coluna 4 e 9: SV65/90 (FCV); coluna 5 e 10: SV534/00 (FeHV-1); coluna 6: SV453/08 (FCV) e na coluna 11: SV555/08 (FeHV-1). A seta no lado direito refere-se ao tamanho de 287 par de bases (bp) correspondente ao fragmento do produto amplificado do FeHV-1 pela PCR (gene TK); e do lado esquerdo, 955 bp corresponde ao produto amplificado do FCV pela RT-PCR (regiões B a F da ORF2). O SV453/08 (FCV) e o SV555/08 (FeHV-1) foram isolados durante a realização da tese.



A



B

FIGURA 4 – Efeito citopático em cultivo celular de origem felina (CRFK) do isolado SV65/90 (FCV) [A] e do isolado SV534/00 (FeHV-1) [B].