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Prospective open pilot study on the use of cam oleum ointment for feline calicivirus disease in cats in Baghdad City

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***Abstract*---**Feline calicivirus (FCV) is a highly contagious virus that causes a mild to severe respiratory infection and oral disease in cats. It is especially common in shelters and breeding colonies, and often infects young cats, this study contained 50 different cats and samples were collected from September 2020 to January 2021. Samples taken by swabs from oropharyngeal and conjunctival area depending on the lesion of FCV infection to investigate viral nucleic acid from collected swabs, then extracting RNA from the swabs and converting it to a cDNA molecule, and last detecting the open reading template gene 2 using specific primer, these samples isolated from veterinary clinics, and shelters, all samples were collected from Baghdad city .Rapid test AG kit and Real time PCR were done to confirm the detection and the results gave us 28 positive results on rapid AG kit and RT-PCR results gave us 32 positive samples and the other 18 samples gave us negative results by detection with nanodrop device before sending all samples for molecular investigation by polymerase chain reaction.

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Most cats recover completely after a calicivirus infection when we treated with Camoleum ointmentprepared in veterinary medicine college lab.

***Keywords*---**FCV Feline Calicivirus, RT-PCR, Laboratory technique, VTM Viral transport media.

Introduction

Feline calicivirus (FCV) is a serious and widespread disease that affects cats. It is a member of the Caliciviridae family, which also comprises several major human and animal infections. As an RNA virus, FCV has a high genome plasticity, which allows it to adjust quickly to selection forces in the environment. This renders the virus extremely adaptive, which has major consequences for clinical disease and its management (Radford, A.D. *et al.*, 2007).

FCV infections are frequently associated with oral ulcers and excessive salivation. (Reubel GH, *et al.*, 1992, Wardely RC *et al.*, 1977).Chronic stomatitis and a limping syndrome are two other clinical syndromes linked to FCV infection (Reubel GH, *et al.*, 1992, Belgard S *et al.*, 2010, TerWee J *et al.*, 1997, Levy JK *et al.*, 1992).Highly virulent systemic FCV infections associated with fatal disease were first reported in North America several years ago (Pedersen NC *et al.*, 2000, Schorr-Evans EM *et al.*, 2003). And later inEurope (Coyne KP *et al.*, 2006, Schulz BS *et al.*, 2011). Outbreaks of severe FCV infections in cats, accompanied by edema and skin ulcerations, have also been reported in Switzerland (Willi et al., 2016).

FCV has also been linked to the upper respiratory tract disease (URTD) complex(Cai Y, et al., 2002, Hoover EA et al., 1975).Also known as "cat flu"In addition to FCV, at least four other pathogens, including feline herpesvirus type 1 (FHV-1), Mycoplasma felis, Chlamydophila felis, and other opportunistic bacteria, have been linked to this syndrome. (Cai Y, *et al.*, 2002, Bannasch MJ *et al.*, 2005, Harbour DA *et al.*, 1991, McArdle HC *et al.*, 1994). Cats with URTD are frequently seen by veterinarians, displaying symptoms such as lethargy, pyrexia, anorexia, sneezing, nasal discharge, ocular discharge, conjunctivitis, and keratitis.(Cai Y *et al.*, 2002, Binns SH *et al.*, 2000).

Material and methods

Real time PCR was used for detection FCV by extraction RNA from infected cats using specific primers as it shown in table 1

Primer preparation

Primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Table 1
primer used in this study

Primer	Sequence	Product (bp)	Reference
F	5' –CAA CCT GCG CTA ACG – 3'	151 bp	GenBank access number M86379.1)
R	5' –TCC CAC ACA GTT CCA AATT – 3'	151 bp	GenBank access number M86379.1)

Rapid test Ag kit:

Commercial lab kitsfor detection the presence of FCV were used by using rapid detection kit (Biotech- Shanghai China) as described in the kit instructions bellow:

Swabs taken from oropharyngeal or conjunctival then put it in the tube contain 2ml of buffer solutions and mixed well then take a small amount of FCV buffer solution then adding from 2 – 4 drops on the FCVAg strip kit then waiting for 5 minutes till the C&T appearance .

Collecting of samples of FCV from cats suffering from oral ulceration & upper respiratory infection

Fifty samples were collected from different cats suffering oral ulceration and respiratory infection during the period from (September 2020 to January 2021). Out of 50 suspected FCV cats, samples were taken from oropharynx and just one sample taken from conjunctiva by a cytobrush swabs and were placed in a tubes containing viral transport media (VTM) then stored in cold box at-4℃ for maximum 4 days or -20℃ for weeks to months until further use (Spiri AM et al 2019).As it shown in the table (4-1) below:

Table (4-1):
The number of Feline calicivirus isolated from different cats

source	No. of total samples	No. of FCV isolates
Oral ulceration & URTDI	50	32

RNA extraction:

RNA Extraction kit (Geneaied, Indonesia) as described in the kit instructions as it showed bellow:

Vortex the preservation tubes containing swabs for 1 minute then added 200 µl of viral transport media (VTM) to a 1.5 ml microcentrifuge tube then Added 400 µl of VB Lysis Buffer to the sample then mix by vortex and added 450 µl of AD Buffer to the sample lysate then shake the tube vigorously to mix and place a VB Column in a 2 ml Collection Tube then transfer 600 µl of the lysate mixture to the VB Column. Centrifuge at 14-16,000 x g for 1 minute and discard the flow-through then place the VB Column back in the 2 ml Collection Tube. Transfer the remaining mixture to the VB Column then Centrifuge at 14-16,000 x g for 1 minute. Discard the 2 ml Collection Tube containing the flow-through then

Transfer the VB Column to a new 2 ml Collection Tube then Add 400 µl of W1 Buffer to the VB Column then centrifuge at 14-16,000 x g for 30 seconds. Discard the flow-through then placed the VB Column back in the 2 ml Collection Tube. Then added 600 µl of Wash Buffer to the VB Column and Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through and place the VB Column back in the 2 ml Collection Tube. Centrifuge at 14-16,000 x g for 3 minutes to dry the column matrix.Place the dried VB Column in a clean 1.5 ml microcentrifuge tube. Added 50 µl of RNase-free Water to the center of the VB Column matrix then let stand for at least 3 minutes to ensure the RNase-free Water is absorbed by the matrix and centrifuge at 14-16,000 x g for 1 minute to elute the purified nucleic acid. Our product was measured through Nanodrop device then converted this RNA samples to cDNA by using Thermo cycler device with first run for 5 minutes at 37ºC and with second run for 1hours at 42ºC and with third run for 5 minutes at 95ºC.

Real Time RT- PCR

Real time PCR was done by using the one step RT-qPCR Master Mix kit (promega, USA)
Each reaction mixture consisted of 5 uL of 2X Master Mix; 0.25 uL of 50X Multiscribe Mix; 0.5 uM of each primer; 0.25 uM of MgCl2 ; 1 uL of template RNA; and 2.5 uL of nuclease deionized free distal water to make a final volume of 10 uL TRT-PCR was performed and Aliquot per single rxn 9µl of Master mix per tube and add 1µl of Template ,PCR Component Calculation : No. of Reaction 4 rxn Annealing temperature of primers 50°C Reaction Volume /run 20 µl No. of PCR Cycles 40. Thermocycling conditions used were:1 cycle of AmpliTaq Initial Denaturation at 95°C for 5 min and 40 cycles of PCR consisting of denaturation at 95°C for30sec and annealing at 50°C for 30sec then Extension at 72°C for 30sec and final extension at 72°C for 7 min then 1 cycle of hold at 10°C for 10min.

CAMOLEUM –ointment

CamOleum ointment was orally used by Applicator contain 5gm of CamOleum ointment used for treatment of oral ulceration caused by FCV and was pushed once daily into the mouth of the patient cats.
The ointment composed from a remarkable 100% natural topical ointment and cream derived from camelid oils (a by-product extracted from camel hump) formulated with other proven effective natural ingredients to create a unique natural topical remedy. Camel hump fat is naturally rich in essential fatty acids Omega 3, 6, & 9 and vitamin E which help to provide optimal conditions necessary for skin regeneration and repair.

Results

Detection of FCV on rapid test Ag kit and by polymerase chain reaction RT_qPCR

The results showed 28positive samples on rapid test cassette and RT-PCR results gave us 32 positive samples and the other 18 samples gave us negative results by

detection of RNA concentration with nanodrop device isolated from veterinary clinics, and shelters, were collected from Baghdad city. FCV detectionin swabsof the oropharynxby RT-qPCR was more likely, compared to using conjunctival swabs, but the direct sampling of an FCV-associated lesion did not increase the likelihood of FCV detection. (Schulz C et al,. 2015). Development of diagnostic nucleic acid amplification techniques has been investigated. Reverse-transcription PCR (RT-PCR) has been developed using a primer set. A real-time PCR for FCV detection has been developed using SYBR Green instead the specific molecular probe (Abd-Eldaim, M. M. 2005).

Nanodrop was used for detection of the RNA concentration and the results gave us 32positive samples and 18 negative samples (>0.1positive, <0.1 negative).Our results on Real time PCR gave us 32 positive samples. As it shown in figure (4-1)

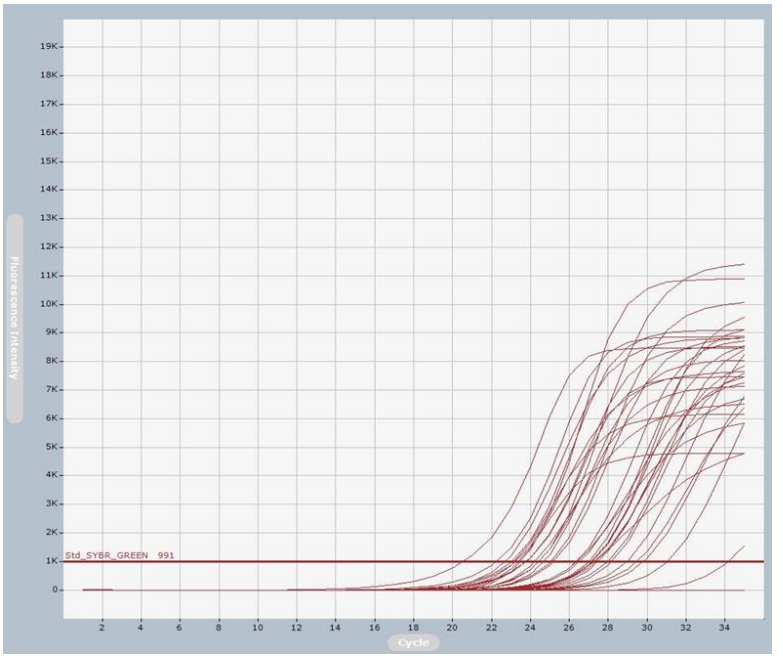

Figure (4-1): cycling curve analysis of the real time-PCR for the tested samples of infected cats

Treatment and Management of FCV Infections

All 50 infected cats with FCV had oral ulcerations and stomatitis in their mouth. CamOleum was used for all their treatments twice daily at the veterinary clinic. There were clinically significant changes between days 0 (before treatment) and 5 after treatment with CamOleum ointment. Cats with mouth and lips dermatitis with eosinophilic plaque of the face had significantly improved with the CamOleum ointment applied to lesioned skin (Figures 1 to 4; Note: these figures are representing typical FCV infections in the 50 cats).

Fig 1: Before andafter 3 days of use cam oleum ointment

Fig 2: Before and after 4 days of use Cam oleum ointment topically on the mouth lesion

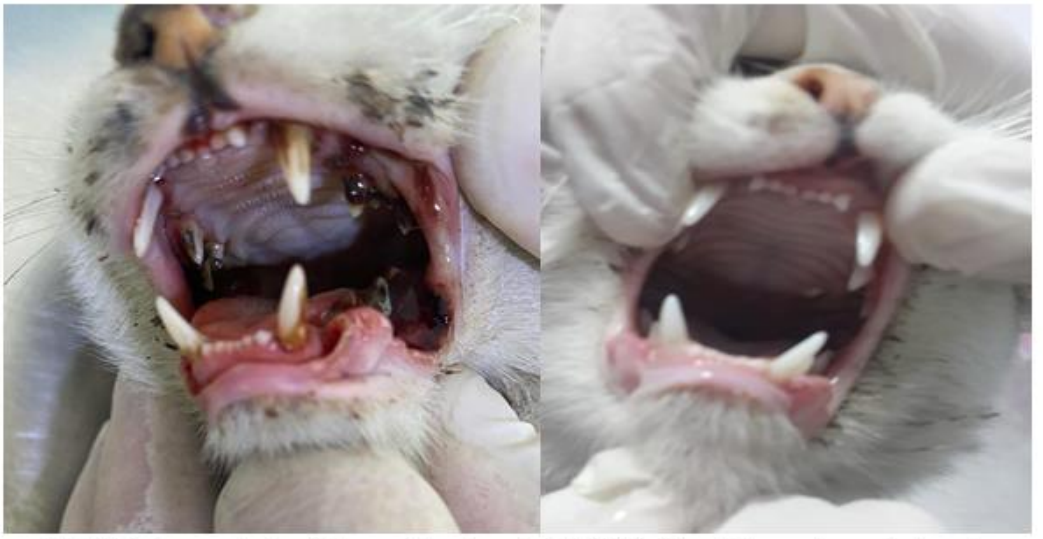

Fig 3: Before and after 5 days of treatment with I.V fluid and Cam oleum ointment topically on the mouth lesion

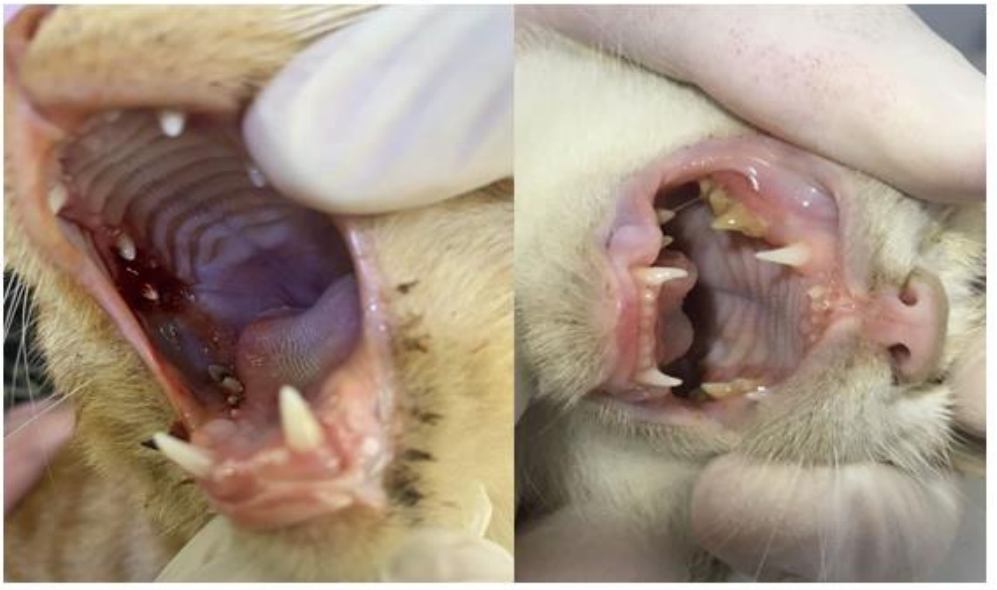

Fig 4: Before and after 2 days of treatment with I.V fluid and Cam ointment topically on lesion once daily

Discussion

Samples for FCV RT-qPCR diagnosis should be transported in viral transport medium, at ≤4 °C, and should be processed as soon as possible, with a maximum of four days of storage. The outcome of sample collection process came in agreement with spiri Andrea Monica (Spiri AM et al 2019). A real-time PCR for FCV detection has been developed using SYBR Green instead the specific molecular probe. The outcome of RT-PCR detection comes in agreement with (Abd-Eldaim, M. M. 2005). In swabs of the oropharynx and the tongue, FCV detection by RT-qPCR was more likely, compared to using conjunctival swabs, but the direct sampling of an FCV-associated lesion did not increase the likelihood of FCV detection this results comes in agreement with Christian Schulz (Schulz C et al,. 2015). Gingivitis, stomatitis, caudal stomatitis, salivation, and oral and lingual ulcerations were significantly associated with the FCV infection (Hoover EA *et al.*, 1975; Cai Y, *et al.*, 2002). This study shows that a topical CamOleum ointment appears to be a highly effective treatment for cats with calicivirus ulcers on the tongue, hard palate, gums, mucous membranes of the mouth, lips, nose and discharge from the nose and eyes serous purulent. In general, all cats appeared after a few days from the treatments with meekness mode, enthusiasm, with significant strength, with approval of food, and drinking, with no trace of FCV. All cats had no diarrhea or constipation. In general, all the signs of lesions with ulcers of the mouth disappeared within only a few days. This suggested that the CamOleum ointment had preference in treating clinical lesions, and the improvement of the cats' quality of life, in which it was found that the efficacy of the treatment by CamOleum ointment is as good or excellent at each time point in the treatment. The response to treatment was rapid, with most of the clinical improvement evident by day 5 to 7. Clinical was tended to improve thereafter in all cats under the trail, and there were significant differences from days 0 up to 7 days in which all lesions showed that all cats were effectively in remission within 1 to 7 days (Figures 1 to 4).The CamOleum ointment treatment was well tolerated, with no adverse events. No significant changes were seen in blood and urine parameters, although only four cats could be sampled. The remaining cats would have required sedation, and it was decided that this would only be performed if the cats' condition gave cause for concern. All cats would tolerate the CamOleum ointment when wiped onto the mouth for treatment and via the insertion of 5 ml of softened CamOleum ointment on the pharynx of the cats. This suggested that the CamOleum ointment could be applied directly to orally treat FCV. The results of this trial indicated that CamOleum has a better benefit and risk profile than other anti-inflammatory agents, such as antihistamines, glucocorticoids and ciclosporin (Latimer et al. 1986; Gregory et al. 1991; Scott et al. 2001; Robson and Burton 2003; Last et al. 2004; Beatty and Barrs 2006; Noli and Scarampella 2006; Vercelli et al. 2006; Nuttall et al. 2009; Wisselink and Willemse 2009; Gharban and Yousif, 2020). This study, however, only followed 50 cats for a maximum of 30 days, and larger and longer-term studies of safety were warranted.This study was carried out to good clinical practice standards (AL-Dulimy and Al-Ani, 2016). Rigorous inclusion and exclusion criteria were established before the trial to ensure, as far as possible, a diagnosis of FCV disease. The diagnosis, however, can be challenging. In particular, it was difficult to carry out the food trials because most cats refused the trial food before completing the 30-day trial. It is therefore possible that some of the cats had

cutaneous adverse food reactions. Selection bias in breed, age, sex, weight, and clinical severity was not apparent. Detection bias by the investigator and owners was, however, likely because this was an open label study. Performance bias was considered unlikely because there were no concomitant treatments apart from flea control. Attrition bias was present, with 5 cats withdrawn, although on-treatment data permitting intention-to-treat analysis were available for all 50 cats. It is possible that this biased towards a favorable response to treatment, although poor efficacy, was cited as a reason for withdrawal in only one case. In conclusion, this study demonstrated that CamOleum ointment was efficacious and well tolerated in 50 cats with FCV disease. It is not, however, licensed for use in cats. This was, furthermore, a small, open label pilot study, and these findings should be confirmed in larger, longer term, blinded randomized, placebo-controlled trials.

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