Isolation and genome sequences of two Feline Morbillivirus genotype 1 strains from Italy

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Veterinaria Italiana 2019, **55** (2), 179-182. doi: 10.12834/VetIt.1847.9883.1
Accepted: 06.06.2019 | Available on line: 30.06.2019

Keywords
Feline morbillivirus, Isolation, Sequencing.

Summary
Feline morbillivirus (FeMV) is a novel viral paramyxovirus detected in cats. FeMV is suspected to be associated to tubulointerstitial nephritis, but its pathogenic role is far to be clearly understood. In this short communication, we report the whole genome coding sequences of the first two FeMV strains isolated in Italy.

Isolamento e sequenziamento del genoma di due ceppi di Feline Morbillivirus genotipo 1

Parole chiave
Feline morbillivirus, Isolamento, Sequenziamento.

Riassunto
Il morbillivirus felino (FeMV) è un nuovo paramyxovirus rilevato nei gatti. FeMV è sospettato di essere associato a nefrite tubulointerstiziale, ma il suo ruolo patogenetico non è stato ancora ben compreso. In questa breve comunicazione sono riportate le sequenze dell'intero genoma dei primi due ceppi FeMV isolati in Italia.

The genus *Morbillivirus* includes several enveloped negative-sense single-stranded RNA viruses infecting humans and animals. Feline morbillivirus (FeMV) is a novel morbillivirus infecting cats and first described in stray cats from Hong Kong nearly ten years ago (Woo *et al.* 2012). Soon after, FeMV circulation was detected worldwide (Furuya *et al.* 2014, Park *et al.* 2014, Sakaguchi *et al.* 2014, Lorusso *et al.* 2015, Sieg *et al.* 2015, Sharp *et al.* 2016, Yilmaz *et al.* 2017, Darold *et al.* 2017).

FeMV isolation on cell culture has been described to be difficult and time consuming (Sakaguchi *et al.* 2014) and a limited number of viral isolates and related whole genome sequences are, indeed, publicly available. Here, we describe the complete genome coding sequences of two FeMV isolates from Italy. Urine samples were taken in March 2018 from two male cats (Tremedino and Pepito, 1 and 7 year old, respectively), living in Reggio Calabria (Calabria region, Southern Italy). The two cats did not show clinical and laboratory signs of renal damage (Donato, manuscript in preparation). Briefly, the first cat (Tremedino, one year old, domestic short-air, male cat) showed stomatitis and an enlargement of popliteal and submandibular lymph nodes, whereas the second cat (Pepito, 7 year old, domestic long-hair male cat) was overweight and presented for stomatitis and bilateral otitis. In both cats, no abnormalities suggestive of renal disease

were recorded at history or physical examination. In the first cat, the haemato-biochemical profile indicated mild eosinophilia [2.85 K/µL, reference range (RR) = 0.17-1.57 K/µL], and severe thrombocytopenia (platelet count 20 K/µL, RR = 300-700 K/µL; low platelet concentration after blood smear examination). Creatinine was 1.1 mg/dl (RR = 0.8-2.4 mg/dL) with serum symmetric dimethylarginine (SDMA) within normal limits [10 µg/dL, reference value (RV) = ≤ 14 µg/dL]. Urine specific gravity (USG) was 1,056 (RV = > 1,035), with a Urine Protein to Creatinine Ratio (UPCR) of 0.09 (RV > 0.4); struvite crystals were also observed. In the second cat, haemato-biochemical profile and urinalysis were unremarkable with serum creatinine (1.0 mg/dL) SDMA (8 µg/dL) and USG (1,038) within normal limits. When urine was collected, an aliquot was immediately 1:8 diluted with MEM for virus isolation. RNA was purified from 280 µL of undiluted urine samples (Biosprint 96 One-For-All-Vet Kit) and tested by a FeMV specific real time RT-PCR (De Luca *et al.* 2018). Resulting C_q values were 35 and 32, for Tremedino and Pepito, respectively. As for virus isolation, five hundred µL of diluted FeMV RNA-positive urine was centrifuged at 3,000 rpm for 5 min to remove debris and filtered through 450 nm disc filters (Millipore). TPCK trypsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was then added to a final concentration of 0.1 µg ml^{-1}. Samples were incubated at 37 °C for 15 minutes. The mixture was then inoculated into feline embryonic fibroblast (FEA) cells in 24-well plates serum-free Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, Zwijndrecht, The Netherlands) supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}) (Invitrogen). After 8 hours, inocula were replaced with MEM (total volume 1 ml) supplemented by 3% heat inactivated fetal calf serum and antibiotics. Cells were incubated at 37 °C in a humidified atmosphere with 5% of CO_2 and observed daily for cytophatic effect by microscopy. At the 1st cell passage, syncytia were evident at day 8. Cells were stained by May Grünwald-Giemsa (Figure 1a). RNA was purified (QIAamp® Viral RNA minikit, Qiagen, Germantown, MD, USA) from 140 µL of cell culture supernatants and tested by real time RT-PCR for FeMV (C_q 26 and 23 for Tremedino and Pepito, respectively).

FEA cells that tested positive by real time RT-PCR were also fixed in chilled acetone at - 20 °C for 20 min. Fixed cells were incubated with 1:100 dilution of rabbit polyclonal antibody against the N protein of FeMV (kindly provided by Dr Shigeru Morikawa, National Institute of Infectious Diseases, Tokyo), followed by incubation with a FITC-goat anti-rabbit IgG (Sigma-Aldrich, Zwijndrecht, The Netherlands) 1:32 diluted. Cells were then examined under a fluorescence microscope and imaged using the Leica TCS SP5 II confocal laser scanning microscope. Uninfected FEA cells were used as negative control. Infected cells tested positive for FeMV (Figure 1b).

Isolates were named FeMV Tremedino/2018 Italy and FeMV Pepito 2018/Italy, further passaged and stored at - 80°. Total RNA was purified from 300 µL of supernatant of the first passage by using the QIAamp® viral RNA minikit (Qiagen, Germantown, MD, USA). Sequencing was performed by using a combination of sequence-independent/single-primer amplification (SISPA) and next generation sequencing (NGS) as previously described (Marcacci *et al.* 2015). Library preparation was carried out by using the Nextera XT Library Prep kit (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Sequencing was

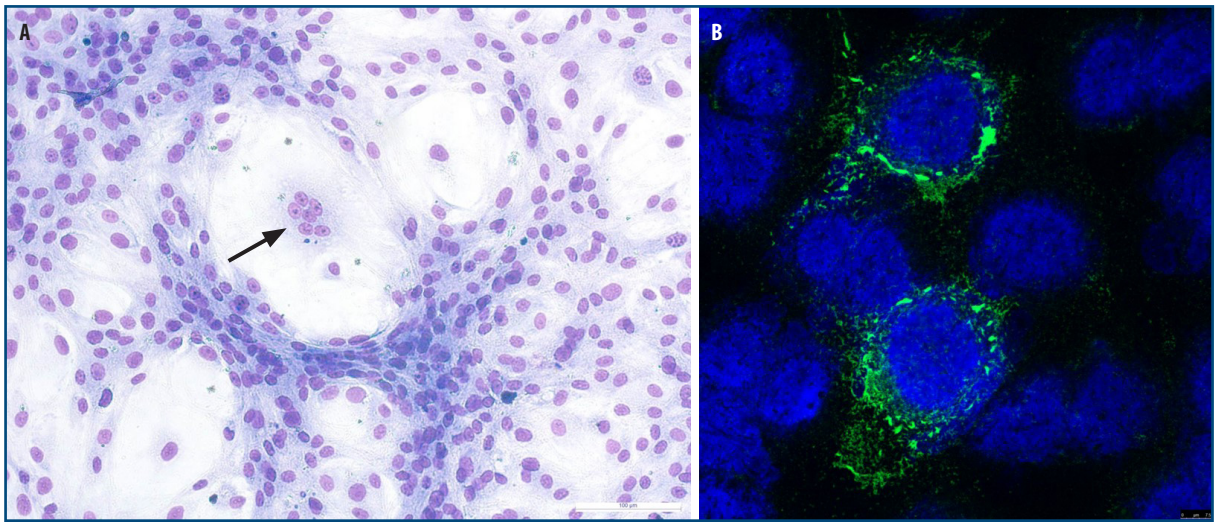

Figure 1. *FEA cells infected by FeMV Pepito2018/Italy.* Multinucleated syncytium was observed by May Grünwald-Giemsa staining (**A**); strong and specific cytoplasmic fluorescence (green color), nuclei are stained with DAPI (blue) (**B**). Scale bar = 100 µm (A), 75 µm (B).

performed on the NextSeq 500 (Illumina Inc., San Diego, CA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles and standard 150 bp paired-end reads. The resulting 3,539,382 and 1,421,904 reads for Tremedino and Pepito, respectively, were *de novo* assembled by SPAdes v3.8.0. A total number of 266,976 and 388,432 reads mapped on a reference FeMV sequence (GenBank accession number AB924120, strain OtJP001). The length of the final *de novo* assemblies were of 16,027 and 15,946 bp for FeMV Tremedino2018/Italy and FeMV Pepito2018/Italy, respectively. The obtained nucleotide (nt) genome sequences were compared to those of extant FeMVs available online and genetic distances were calculated by using MegAlign (Lasergene 15.0, Madison, WI, USA). The genome sequences of Tremedino2018/Italy and Pepito2018/Italy were found to be nearly identical as they share the 99.2% of nt identity. Nt identity between sequences obtained in this study and extant whole FeMV genome sequences ranges from 98.7% to 78.1%. Tremedino2018/Italy and Pepito2018/Italy showed the highest % of nt sequence identity with the Japanese strains SS1 (98.7%, AB910309) and OtJP001 (98.5%, AB924120); nt identity was lower with the early FeMV strains 761U and 776U (87.8%, JQ411014 and JQ411015) isolated in Hong Kong (Woo *et al.* 2012) and with strain US1 from USA (87.9%-87.8%, KR014147). Tremedino2018/Italy and Pepito2018/Italy strains share the 88.1% of nt identity with Piuma/2015, the first FeMV strain described in Italy in 2015 (Lorusso *et al.* 2015). Very recently, a new genotype of FeMV, tentatively named FeMV genotype 2 (FeMVGT2), was described in Germany (Sieg *et al.* 2019). FeMV sequences obtained in this study share the 78.1% of nt identity with FeMVGT2 sequences. Overall, our results confirm the viral heterogeneity existing between FeMV circulating strains and that the strains described in this study belong to the FeMV genotype 1. Further molecular analysis of FeMV strains circulating in Southern Italy is currently underway (Donato, manuscript in preparation) as well as the assessment of a serum-neutralization assay to quantify specific FeMV antibodies in cat serum.

Nucleotide sequence accession numbers

Nucleotide sequences of Tremedino2018/Italy and Pepito2018/Italy have been deposited in GenBank with accession numbers MK088516 and MK088517, respectively.

Acknowledgements

Funding were provided by the Italian Ministry of Health (MSRCTE06/17, Ricerca Corrente 2017 "Nuovi flussi diagnostici in Sanità animale: dalla NGS alla banca antigeni", recipient Alessio Lorusso). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo Italy.

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