Feline morbillivirus in northwestern Italy: first detection of genotype 1-B

Genotype 1-B of Feline Morbillivirus in north-western Italy without evident relationship with kidney diseases

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SUMMARY

Objectives: A novel Morbillivirus was recently described in stray and domestic feline population of Asia, America and Europe. Most cats infected with Feline Morbillivirus (FeMV) are affected by low urinary tract or kidney's disease. However, although association of FeMV infection with kidney diseases in cats has been suggested, the pathogenicity of the virus remains unclear. The present study aimed to investigate the distribution of FeMV infection as well as the relationship between FeMV infection and kidney diseases in cats of north-Western Italy.

Methods: A total of 153 samples of urine (150 individuals and 3 pools) and 50 samples of kidneys were collected and included in the present study; total RNA was extracted and a reverse transcription-quantitative PCR (rt-qPCR) was performed in order to identify FeMV. Kidneys were also submitted to anatomopathological investigations. Phylogenetic analysis and isolation attempts were carried out on positive samples. In FeMV positive cats' urinalysis and blood analysis were performed.

Results: FeMV RNA was detected in 7.3% of urine samples and in 8% of the kidneys both in healthy cats and in cats with clinical signs or post-mortem lesions compatible with kidneys' disease. At histopathological examination kidneys showed tubule-interstitial nephritis (TIN) in three out of four positive samples, but a clear relationship between FeMV infection and TIN was not observed. Isolation attempts resulted unsuccessful, although the urine sample of one castrated male cat hosted in a cattery showed a positive signal in rt-qPCR until the fourth cell passage. Interestingly, in the same cattery a second variant was detected from a urine pool. Urinalysis showed proteinuria in all cats while blood analysis reported altered creatinine levels in two positive cats.

Conclusion and Relevance: Data reported suggests the presence of a FeMV sub-cluster well distinct from the strain previously isolated in Italy whose role in renal disorders remains uncertain.

INTRODUCTION

The genus *Morbillivirus* includes a consistent number of pathogens that cause some of the most devastating viral diseases of humans and animals (i.e. Measles virus, Canine Distemper virus, Rinderpest virus)[1]. In 2012 a novel Morbillivirus has been discovered in stray cats' population of Hong Kong and named Feline Morbillivirus (FeMV)[2]. FeMV is a negative sense not-segmented single stranded RNA virus whose genome of 16.050 bp was recently characterized[3]. It follows the "rule of six", typical for morbilliviruses: 6 genes (N, P/V/C, M, F, H, L) coding for six structural and two non-structural proteins. A not negligible number of FeMV infected cats in Japan showed lower urinary tract diseases (LUTD) with typical tubulointerstitial nephritis (TIN)[2] but possible correlations between renal diseases and FeMV infection remain still unclear[4]. In recent years, different epidemiological studies were carried out in Japan, United Kingdom, Brasil, Turkey, and Italy[3,5–8]. From a diagnostic point of view, several biomolecular approaches were developed and optimised for investigation purposes[5,6,8–10]. Previous studies demonstrated that urine and kidney represent the best targets for biomolecular detection of FeMV by rt-qPCR [11,12]. Concerning isolation procedures, FeMV was demonstrated to replicate into Crandell-Rees feline kidney cells (CRFK)[13], and into feline embryonic fibroblast (FEA) cells[14] causing a discrete cytophatic effect characterised by syncytia formation. However, long incubation periods for the infected-CRFK and biological features of FeMV make isolation attempts time-consuming and often unsuccessful[12]. Indeed, Koide et al., (2015)[13] demonstrated that viral titre increases exponentially between the 18th and the 30th hour post-inoculation on CRFK. Conversely, no increasing in virus titre was observed after 63 hours post-infection[13]. However, only few studies

on FeMV reported information about cat anamnesis or clinico-pathological features related to FeMV and data regarding the correlation between kidney diseases and FeMV infection are often discordant. The aim of this study was to investigate the presence of FeMV as well as the relationship between FeMV infection and kidney disease in cats of North-Western Italy performing biomolecular analysis on kidney and urine samples and histopathological examination of kidney. Phylogenetic analysis was finally carried out to investigate genetic correlations with other field strains from different countries.

MATERIALS AND METHODS

SAMPLES COLLECTION

Urine

A total of 150 individual urine samples were collected from cats hospitalised in a veterinary clinic or hosted in a private cattery. Particularly, 127 client-owned cat and 23 cats from the cattery were sampled. The private cattery hosted a total of 55 cats at the time of the study divided in 4 rooms with an adequate number of food and water bowls, litter boxes and cat beds. All the rooms had access to an external fanced area where the cats were free to move. No cats were kept in cages. Moreover, in the same cattery, three pooled urine samples were collected in order to rapidly screen the hosted population for FeMV as some cats of the cattery are feral cats and cannot be manipulated without sedation. Urine pools were collected confinating 10 cats per room and placing different litter boxes in each room. All individual cats (n=150) included in the study were clinically examined and sex, breed and age were recorded (Table 1). Concerning sex ratio, 68% of the cats were male, mainly neutered, while 32% were female, mainly spayed. Urine samples were collected from animals of ten different breeds (Table 1). Across the animals included in this study, 21% of cats were affected by LUTD; acute and chronic urinary failures were detected

in 3% and 25% of cats, respectively. The remaining cats (51%) were healthy or affected by non-urinary diseases. Urine samples were kept frozen at -80°C until the extraction of total RNA.

Kidneys

Fifty renal tissue samples were collected from cats necropsied at the Department of Veterinary Science (University of Turin, Italy). Particularly, 40 kidney samples were from client-owned cats and 10 kidney samples were from cattery cats. Sex, breed, age and available anamnestic information were gathered (Table 1). Six different breeds were included. Concerning sex ratio, 50% were male, mainly and 50% were female, mainly unspayed. Both gross and histopathological investigations were performed in order to describe *post-mortem* features of the tested kidneys. Moreover, kidney samples were kept frozen at -80°C until total RNA extraction.

BIOMOLECULAR ASSAYS

Total RNA was extracted from 350µl of urine or 1 mg of renal tissue by using Trizol RNA isolation reagents (Life Technologies, Carlsbad, CA) following the manufacturer's instructions with a final elution in 30 µl DNase/RNase-free water. The extracted RNA was tested by One Step rt-qPCR according to De Luca et al., (2018)[12], with minor modifications. The diagnostic assay target was represented by a fragment of the P/V/C gene (74 bp) and the original protocol was optimized for use with Superscript III Platinum One Step rt-qPCR System (Invitrogen, Carlsbad, CA). The reaction volume of 25µl contained 5µl of purified total RNA, 12.5 µl of Superscript III One-Step rt-qPCR Invitrogen Reaction Mix, a final concentration of 0.6 µM for each primer (FeMV-rt Forward 5′- GGGATCCAGAGGGTAACCT -3′ and FeMVrt Reverse 5′-CCGGCCATTAATCTCTGAA -3′), 0.225 µM of FeMVrt TaqMan Probe (5′-CCGGCCATTAATCTCTGAA -3′), 4 µM of $MgSO_4$, 0,5µl of Taq mix and nuclease-free water up to the final volume. The assay was carried out on a CFX96 Touch™ rt-PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA), setting the following thermal conditions: 1 cycle of

reverse transcription at 50°C for 30 min, 1 cycle of PCR initial activation step at 95°C for 15 min followed by 45 cycles at 95°C for 30 s, 55 °C for 40 s and 60°C for 30 s.

FeMV ISOLATION ATTEMPTS

From each rt-qPCR positive sample, FeMV isolation was carried out according to Woo et al. (2012)[2]. Urine samples were diluted 1 to 10 into Minimum Essential Medium Earle (MEM-Earle, Biowest, Riverside, MO) supplemented with a solution 5X of a stock 1000X of penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}) (Sigma–Aldrich, St. Louis, MO). The mixture was filtered through 450 nm disc filters (Millipore), further diluted 1 to 2 in MEM Earle supplemented with 1µg/ml of Polybrene Infection Reagent (Sigma-Aldrich, St. Louis, MO) and inoculated into a flask of 25cm^2 of CRFK cells. After 1 hour of incubation in gentle agitation at 37°C, the mixture was decanted and the CRFK were washed two times with PBS 1X. Seven ml of MEM supplemented with 0,1 µg/ml of *L*-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK)-treated trypsin and with 2% heat-inactivated fetal bovine serum (Gibco, Thermofisher scientific, Waltham, Massachusetts) and antibiotics (see above) were added directly on the monolayer. The CRFK were incubated at 37 °C in a humidified atmosphere with 5% of CO_2 and observed daily for cytophatic effect (CPE) by microscopy. Each passage consisted of twelve days of incubation then the cell lysate was tested by FeMV rt-qPCR protocol[12] and further cells passages were performed until the cell lysate stayed positive in biomolecular investigations. The Cq mean values of cell passages were compared in order to semi-quantitatively detect a potential increase in virus titre, indicative of efficient viral replication.

MORPHOLOGICAL AND HISTOPATHOLOGICAL INVESTIGATIONS

Systemic post-mortem examination was performed on the cats, and kidneys were grossly evaluated. All the kidneys also underwent histopathological investigation in order to confirm macroscopic diagnosis. Cross-sections of renal cortex, medulla and pelvis were collected, fixed

142 in 10% buffered formalin solution, paraffin embedded, sectioned using a microtome, and stained
143 with hematoxylin and eosin (HE). The samples were observed by means of light microscopy.
144 The histopathological evaluation was mainly focused on the presence of acute or chronic TIN
145 with associated glomerulopathies, sclerosis, calcifications, granulomas or tumor lesions.

146 **URINE AND BLOOD ANALYSIS**

147 Urinalysis was performed in rt-qPCR positive samples in order to try to correlate FeMV with
148 urinary tract infections and subsequent altered urine composition as reported by Yilmaz et al.,
149 (2017)[8]. Samples were obtained by cystocentesis or spontaneous urination. Each sample was
150 placed in a sterile universal tube and processed for physical and chemical analysis within 6
151 hours after sampling. Urinalysis was performed using dipstick tests (Multistix-10-SG, Siemens
152 Healthcare Diagnostics, Tarrytown, NY) read by Clinitek Status analyzer (Siemens Healthcare
153 Diagnostics, Tarrytown, NY). Urine specific gravity (USG) was measured by refractometer.
154 Microscopic examination of the urine sediment on 10 field at 100x magnification was also
155 performed in order to identify casts, crystals or epithelial cells. Moreover, two blood samples
156 were collected in positive cats: the first one was collected with urine samples (T0) and the
157 second one was collected one month later (T30). Blood samples were placed in sterile tubes to
158 evaluate blood creatinine (mg/dl) as a marker of renal function according to IRIS (International
159 Renal Interest Society) guideline[15].

160 **PHYLOGENETIC ANALYSIS**

161 The total RNA of PCR positive samples was reverse transcribed by First Strand cDNA
162 Synthesis Kit (Roche, Basilea, Switzerland). The cDNA was subsequently used for the
163 amplification of a fragment (401 bp) of L gene using a double-step nested PCR, as described by
164 Furuya et al. (2014)[5].

An agarose gel electrophoresis allowed to detect nested-PCR products with GelGreen® Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA) using Uvitec technology for image capture. The amplified fragment of 401 bp was sequenced after DNA purification from agarose gel, performed by High Pure PCR Product Purification Kit (Roche, Basilea, Switzerland). The Sanger sequencing was conducted using the BigDye® Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA). Two software were used for sequence analysis of the detected basis (Sequencing Analysis) and for the manual correction (FinchTV, Geospiza, Inc), respectively. From the obtained sequences of the amplified products, the primer sequences were removed and the software Clustal Omega[16] allowed the alignment with 30 sequences of FeMV genotypes available in the public database NCBI, and 3 sequences from Lombardy[17], kindly provided by Dr. Stefania Lauzi (Università degli Studi di Milano Statale, Milan, Italy). For phylogenetic inference, the best nucleotide substitution model was estimated by jModelTest2[18]. The phylogenetic tree was constructed by MrBayes v. 3.2.6[19] with the following settings: 2 parallel runs of 4 chains each for 5,000,000 generations and TPM1uf+Ias nucleotide substitution model, as selected by jModelTest2, and a 10% burn-in to summarize parameter and tree log files. Convergence and effective sample size (ESS) were controlled with Tracer v1.7.1. The tree was then visualized with Figtree v1.4.4.

RESULTS

Biomolecular assays

Eleven out of 150 individual urine samples (5 client owned cats and 6 cats from the cattery) and two urine pools resulted positive for FeMV RNA according to the cut-off level established by Lorusso and colleagues (2015)[10]. As for kidney samples, 4 out of 50 (3 client owned cats and 1 cat from the cattery) were positive for FeMV RNA (Table 1).

FeMV isolation attempts

The entire panel of RT-Real-Time positive samples were used for isolation attempts; nevertheless, just the urine sample of “cat Totò” showed a promising biomolecular outcome for isolation purposes. The first two cell passages, tested by rt-qPCR showed Cq mean values (23.48 and 24.22 respectively) higher than the starting urine sample (29.78). However, no FeMV-associated cytophatic effect was observed during 13 days of daily observations. Conversely, isolation attempts from the remaining positive samples (4 kidney and 11 urine samples) were unsuccessful from the first cell passage.

Morphological and histopathological investigations

At gross examination, 20 cats showed no macroscopic detectable lesions (40%), while 30 cats showed different renal injuries (60%). In particular, pale firm hypertrophic/hypotrofic kidneys, multifocal cortical granulomas and renal infarcts were detected.

At histological examination, TIN was detected in 50% of processed samples (Figure 1A), while granulomatous nephritis (Figure 1B) and tumor lesions were detected in 14% and 4% of examined cats, respectively. Finally, 16% of the analyzed kidney samples showed other lesions (tubular calcification, hyperemia, tubular steatosis, metastasis)and 16% did not present any histopathological lesions (Table 1).

Among the four rt-qPCR positive kidneys, one cat showed no detectable lesions at gross examination, one cat had pale firm hypotrophic kidneys while two cats presented bilateral hypertrophy and multifocal cortical granulomas. Histopathological findings revealed the presence of TIN in three out of four animals, while the last cat was affected by a systemic lymphoma with evident renal metastatic lesions.

Urine and blood analysis

Among the 11 cats with rt-qPCR positive urine, 9 samples were available for urinalysis (4 cats from the cattery and 5 client owned cats). In particular, 8 cats showed normal urine pH (6-7) while one showed acidic urine (pH 5); 6 showed normal USG (⩾ 1035) while 3 presented lower USG (1014-1027). Three cats presented proteinuria and 7 showed urine sediment with crystals, epithelial cells and lipid droplets. Regarding blood creatinine levels, the two blood samples were used to classify cats creatinine levels according to the IRIS guidelines[15]:7 cats were classified as IRIS stage 1 (<1.6 mg/dL), 1 cats was IRIS stage 3 (3.61 mg/dL) and 1 cats was IRIS stage 4 (12.38 md/dL) (Supplementary Table S1).

Phylogenetic analysis

Across the entire set of rt-qPCR positive samples, sequencing of a portion of FeMV L gene (401 bp) was possible only for two of them. In more details, the first sequenced sample was obtained by urine of cat Totò collected by cystocentesis (GenBank Acc. Num. MT561453); the second sequenced sample was obtained by a pool of urine collected from cats cohabitant with Totò (GenBank Acc. Num. MT561453). The percentage of sequence similarity between the two samples was calculated as 97.9%. Moreover the two sequences belong to the clade of the German isolates Schmusi and Moj, but they are phylogenetically distant from the first Italian isolate of cat Piuma and all the other Italian strains[3,17]. Focusing on the Italian samples, they are present in three out of four sub-clusters (B, C, and D) Sub-cluster D is composed by Italian samples only. Ours are the only Italian samples in sub-cluster B (Figure 2).

DISCUSSION

FeMV represents a novel negative single-stranded RNA virus with still few information about dynamic of infection, host susceptibility in target population, time and ways of excretion by infected animals. After its first discovery in Hong Kong[2], FeMV was reported in many other

236 countries with different prevalence in urines ranging from 3% to 23% in the US[9] and Japan[4],
237 respectively.
238 In 2015, FeMV was identified in Italy from the urine of a 15-years old stray cat with suspected
239 chronic kidney disease (CKD)[10], and recently Stranieri et al. (2019)[17] investigated the presence
240 of FeMV in Northern Italy reporting a lower prevalence (1.23%) compared with previously
241 published studies.
242 According to in-vitro studies, the ability of the virus to cross species barrier and to infect humans
243 seems to be remote[20]. However, new epidemiological, histopathological and phylogenetic
244 investigations could improve field strains' characterization by increasing current knowledge
245 about this new member of the genus Morbillivirus. Our data show that at least three different
246 sub-clusters of FeMV are circulating in Italy, and the samples collected in our study do not
247 share the same clade with the other Italian strains. Hence, a multiple introduction of the virus
248 may have occurred in Italy, but further analyses, especially at genomic level, are needed.
249 In this scenario, this study focused on different diagnostic aspects of FeMV infection:
250 biomolecular detection in biological samples, isolation attempts from rt-qPCR positive matrices
251 (both urine and kidney samples) and sequencing of target genes in order to reveal phylogenetic
252 relationships.
253 Considering the group of cats involved in the present study, the rate of infection appears
254 relatively low (7.3 %) but quite similar to the prevalence data reported in the literature,
255 particularly in stray cats, believed more easily infected than client-owned[2]. The case of “cat
256 Totò” and of the cohabitant cats from the private cattery (mostly negative for FeMV detection)
257 leads to speculate that just few animals can develop an effective infection and/or an effective
258 excretion of FeMV. Moreover, according to the detected sequence data, a second FeMV strain,
259 different from Totò's strain, was present in the cattery. Even though other 5 cats cohabiting with

Totò resulted positive to the biomolecular assays, it can be hypothesized that cat-to-cat transmission is not a common occurrence, or that FeMV infection could have a long incubation period before viremia and viral shedding[8].

A serological investigation could clarify how many cohabitant cats have developed at least a humoral response against FeMV but, unfortunately, no commercial serological ELISA is currently available for this purpose. Moreover, the development of an “in house ELISA” would require a considerable number of antibody-positive FeMV sera, which are actually not available as well. Concerning clinical features of FeMV infection, cat Totò showed no signs of renal disease or LUTD and was healthy for the entire study. Regarding urine positive samples, two cats were affected by CKD, while the remaining FeMV positive cats were negative to renal disease and all cats presented proteinuria. These finding are in agreement with a recent study reporting not clear correlation between chronic urinary disease and FeMV infection[8].

The authors investigated also a possible association between FeMV infection and TIN. TIN was detected in three out of four RT-Real Time positive kidney samples, and this feature could support the hypothesis of a co-existence of FeMV infection with renal disease.

On the other hand, the presence of FeMV viral RNA in one kidney affected by lymphoma could suggest that FeMV is possibly able to cause lesions other than TIN. Clinically only one positive cat was affected by CKD. Moreover, in the present study, TIN was detected in the 54% of all processed kidneys, and granulomatous nephritis was observed in the 14%. Therefore, a clear relationship between the presence of FeMV infection and kidney diseases in cats was not observed according to recent studies[5,7,8,17,21].

Concerning host susceptibility in cats, few information is currently available about viral absorption or mechanisms involved in feline viremia but it is likely that just certain animals expose optimal receptors involved in viral infection and/or in virus excretion. A panel of

candidate cell-receptors should be evaluated at molecular level in order to identify sequence mutations associated with more efficient FeMV membrane transport[22].

CONCLUSION

In conclusion, this is the first study reporting FeMV genotype 1-B in North-western Italy suggesting that at least three different sub-clusters of FeMV are circulating in Italy (subcluster B, C and D). Further studies are required to better investigate the potential role of FeMV in the pathogenesis of kidney disease.

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Conflict of interest

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Ethical approval

This work involved the use of non-experimental animals only (including owned or unowned animals and data from prospective or retrospective studies). Established internationally recognised high standards ('best practice') of individual veterinary clinical patient care were followed. Ethical approval from a committee was therefore not necessarily required.

Informed consent statement

Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work (either experimental or non-experimental animals) for the procedure(s) undertaken (either prospective or retrospective studies). No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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Figure Legend

Figure 1. A) Cat, kidney. Multifocal moderate interstitial nephritis. Haematoxilin and Eosin (H-e), 100x. B) Cat, kidney. Multifocal moderate granulomatous nephritis. H-e, 100x.

Figure 2. Bayesian phylogenetic tree based on a 339 bp long fragment of L gene of the Feline morbillivirus. Node support is reported as posterior probability for the node. The strains characterized in this study are marked as bold. Genotype and sub-cluster of the clades is reported as described by Sieg et al (2019) and Stranieri et al (2019). The novel clade described by Stranieri et al (2019) has been named here as genotype 1-D, in order to continue the previous nomenclature.

Table 1. Signalment, clinical signs and test results of urine samples analyzed in this study

	URINE					
	N	rt-qPCR positive	Clinical diagnosis			
			FLUTD	CKD	AKD	Other[1]
	150					
Age (years)						
< 2	5	0	3	0	0	2
2-9	93	8	21	22	3	47
>10	52	3	8	14	2	28
Sex						
Castrated male	88	7	21	23	3	41
Uncastrated male	14	0	5	0	1	8
Spayed female	44	3	6	12	1	25
Unspayed female	4	1	0	1	0	3
Breed						
Cross	122	11	28	27	4	63
Pure	28	0	4	9	1	14
Birman	4	0	1	2	0	1
British short hair	4	0	0	4	0	0
Chartreux	3	0	0	0	1	2
Exotic short hair	1	0	0	1	0	0
Maine Coon	2	0	0	0	0	2
Norwegian Forest	4	0	0	0	0	4
Ragdoll Seal Point	3	0	2	0	0	1
Persian	4	0	1	1	0	2
Siamese	3	0	0	1	0	2
Siberian	0	0	0	0	0	0

FLUTD: feline lower urinary tract disease; CKD: chronic kidney disease; AKD: acute kidney disease; [1] pathology not related to urinary tract;

Table 2. Signalment, histopathological findings and test results of kidney samples analyzed in this study

	KIDNEY						
	N	rt-qPCR positive	Histological findings				
			Lymphoma	TIN	GN	Other[1]	ALS
	50						
Age (years)							
< 2	10	0	0	2	3	3	2
2-9	25	3	2	10	4	3	6
>10	15	1	0	13	0	2	0
Sex							
Castrated male	12	1	0	8	2	0	2
Uncastrated male	13	0	0	7	3	1	2
Spayed female	4	2	0	2	0	1	1
Unspayed female	21	1	2	8	2	6	3
Breed							
Cross	42	3	2	22	5	8	5
Pure	8	1	0	3	2	0	3
Birman	0	0	0	0	0	0	0
British short hair	0	0	0	0	0	0	0
Chartreux	3	0	0	2	0	0	1
Exotic short hair	0	0	0	0	0	0	0
Maine Coon	2	0	0	0	2	0	0
Norwegian Forest	1	1	0	1	0	0	0
Ragdoll Seal Point	0	0	0	0	0	0	0
Persian	1	0	0	0	0	0	1
Siamese	0	0	0	0	0	0	0
Siberian	1	0	0	0	0	0	1

TIN: Tubulo-intesrtitial nephritis; GN: granulomatous nephritis; ALS: absence of alterations. [1]tubular calcification, hyperemia, tubular steatosis, metastasis.