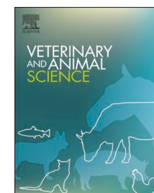
Veterinary and Animal Science 6 (2018) 75–80

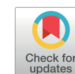
Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas



Research on viral agents associated with feline reproductive problems reveals a high association with feline panleukopenia virus



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ARTICLE INFO

Keywords:
Loss of pregnancy
Felis catus
Reproductive failures
Infectious causes

ABSTRACT

Although reproductive failures (RF) such as abortion, stillbirth and neonatal mortality in cats are still under researched, it is known that many RF are caused by viral agents. This research surveyed the viral agent prevalence in queens with RF. Queens were excluded from the study if their RF was caused by issues other than infection, such as genetic, traumatic, hormonal or nutritional problems, or if they had a history of RF. Blood samples from 26 pregnant females with RF were collected for complete blood counts (BCC), renal/hepatic biochemistry and glycaemic analysis. Ultrasonography was performed to evaluate gestational age and foetal viability. When possible, placentas, humours and foetal tissues were collected. Blood samples were tested by PCR and qPCR for feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), *feline alphaherpesvirus 1* (FeHV-1) and *carnivore protoparvovirus 1* (CPPV-1). All maternal samples were negative for FeLV, FIV and FeHV-1 and positive for CPPV-1. In addition, foetuses from one queen and three females were positive for CPPV-1 by qPCR and for feline panleukopenia virus (FPV) through DNA sequencing. The BCC and biochemistry results revealed significant neutrophilia, lymphopenia, monocytosis, and liver enzymes. These results provide the first description of an FPV agent causing only RF-related clinical signs in queens.

1. Introduction

Reproductive failures (RF) such as abortion, stillbirth and neonatal mortality are common during feline pregnancy, with infectious agents playing important roles in determining the outcomes (Romagnoli, 2003). Few studies have been conducted on infertility and pregnancy loss in cats (Verstegen, Dhaliwal, & Verstegen-Onclin, 2008); however, viruses are known to be a significant cause of feline RF. In these cases, RF may be transmitted via the placenta, directly infecting the embryos and foetuses, or by severely debilitating pregnant animals in the absence of congenital infection (Decaro, Carmichael, & Buonavoglia, 2012). Some members of the genus Protoparvovirus (PV) are important pathogens in domesticated cats and dogs, particularly in young animals (Cotmore et al., 2013; Tijssen et al., 2011). Canine parvovirus (CPV) is a deadly enteropathogen that is fatal in puppies and adult dogs worldwide (Decaro & Buonavoglia, 2012). CPV has recently been included in the same viral species as the carnivore protoparvovirus 1 (CPPV-1), feline panleukopenia virus (FPV), and wild carnivore parvoviruses, which cause many diseases, particularly in foetal and newborn animals (Cotmore et al., 2013,2014).

FPV is a small, non-enveloped single-stranded DNA virus that infects domestic cats and other Felidae as well as species in the families Mustelidae, Procyonidae, and Viverridae including racoons, ring-tailed cats, foxes and minks (Stuetzer & Hartmann, 2014). The virus causes feline panleukopenia (FP), a disease characterized by severely reduced circulating white blood cell counts (WBCC) and enteritis with degeneration of the intestinal villi. Infection is highly contagious and is associated with high mortality and morbidity. In utero infection in early pregnancy can result in foetal death, resorption, abortion, and mummified foetuses, and in later pregnancy, FPV can cause damage to neuronal tissue (Csiza, Scott, de Lahunta, & Gillespie, 1971; Stuetzer & Hartmann, 2014). Clinical symptom severity depends on age, immune

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https://doi.org/10.1016/j.vas.2018.06.004
Received 1 March 2018; Received in revised form 22 May 2018; Accepted 19 June 2018
Available online 02 July 2018
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status, and concurrent infections; not all cats infected with FPV develop clinical signs of panleukopenia (Foley, Orgad, Hirsh, Poland, & Pedersen, 1999).

Other CPPV-1 species also affect cats, including the new variants of CPV: CPV-2a, 2b (Decaro et al., 2011; Truyen et al., 1995) and 2c (Battilani, Bassani, Forti, & Morganti, 2006; Decaro et al., 2010), which infect and cause diseases that are indistinguishable from FPV. However, FPV is the most prevalent strain of protoparvoviruses to cause disease in cats. Furthermore, since cats are susceptible to both CPV-2 and FPV viruses, superinfection and coinfection may occur, facilitating recombination and high genetic heterogeneity (Battilani, Scagliarini, Ciulli, Morganti, & Prosperi, 2006; Hoelzer, Shalckelton, Holmes, & Parrish, 2008; Mochizuki, Harasawa, & Nakatani, 1993). The role of these variants in cats remains uncertain. These new variants of CPV have shown to be less frequent in healthy cats (Mochizuki et al., 1993; Whitby et al., 2010). Phylogenetic analyses of these viruses demonstrated a high sequence similarity to CPV sequence types 2a and 2b in clinically ill dogs (Whitby et al., 2010). In addition, some experimentally infected cats with CPV only show haematological abnormalities (Chalmers, Truyen, Greenwood, & Baxendale, 1999).

Among the most commonly reported feline viral agents are feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), feline alphaherpesvirus 1 (FeHV-1) and feline panleukopenia virus (FPV) (Verstegen et al., 2008). There are four FeLV subtypes that can cause abortion, infertility and foetal resorption (Verstegen et al., 2008). Cats do not often present symptoms prior to abortion (Troy & Herron, 1986). FIV can be transmitted in utero, resulting in miscarriage, stillbirth, and paused or retarded foetal development, but may also lead to the birth of viable kittens infected with the virus. Experimental inoculation with FIV in free-of-pathogen cats resulted in 60% of the foetuses being reabsorbed or exhibiting delayed foetal development (Weaver et al., 2005). FHV-1 caused abortion and intrauterine foetal death (Johnston, Kustritz, & Olson, 2001). However, the virus was not isolated from the aborted foetal tissues (Smith, 1997). This pathogen appears to cause miscarriage due to a debilitating upper respiratory infection in cats and can also infect kittens during the neonatal period (Verstegen et al., 2008).

Accounting for the CPPV-1 significance in felines and the relevance of cats with a history of difficult delivery, miscarriage, stillbirth and neonatal and foetal mortality, this study examined the prevalence of FeLV, FIV, FHV-1 and FPV that commonly affect domestic queens with RF.

2. Materials and methods

2.1. Sampling

This study was conducted in 2015 in accordance with the Committee on Ethics in Animal Use (CEUA) - UFERSA (n° 23091.006795/2015 - 88). The animals were included after their owners signed a consent form and answered a questionnaire (adapted from Lamm & Njaa, 2012). The queens who were used in the study had no history of genetic, traumatic, hormonal or nutritional problems, nor a history of previous pregnancy problems. A total of 42 blood samples were collected from the queens (26 with RF and 16 without RF) at the Veterinary Hospital Jerônimo *Dix-Huit* Rosado at the Federal Rural Semi-Arid University (UFERSA) in Mossoro, RN, Brazil.

Blood samples from the queens with and without RF were collected by venipuncture of the jugular vein in two vacuum container tubes, one with and one without EDTA. A complete blood count (BCC), biochemistry of the kidneys and liver, and serum glucose tests were performed. The animals were considered to have an infectious leukogram when the WBCC was outside the reference values (reference 7–23 mil/mm^3) and considered to be anaemic when the red blood cell (RBC) values, haemoglobin (8–15 g%) and haematocrit (24–45%) were below reference values (5–10 mil/mm^3). Hypoglycaemia was considered when the glucose values were below the reference range (70–110 mg/dL), and the presence of kidney disease was considered when the biochemical values of urea and creatinine were above the reference ranges (43–64 mg/dL and 0.7–1.7 mg/dL, respectively). Liver disease was considered when the biochemical values of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were above the reference ranges (10–50 U/L and 10–40 U/L) (Meyer & Harvey, 2004).

Ultrasonography was performed to identify gestational age and foetal viability. Samples from the 26 queens with RF were collected in the operating room after they were admitted for ovariosalpingohysterectomy (OSH) or caesarean section. Macroscopic examinations of the uterus (normal or haemorrhagic), placenta (normal or haemorrhagic) and foetuses (normal, foetal death/mummification or haemorrhagic) were performed and photodocumented. From the queens, we collected blood to obtain serum and stored the whole blood and uterus/placentas. From the foetuses, all organ tissues were collected depending on the condition of the material (mummified, macerated or not). All samples were stored in a freezer at −20°C until complete DNA extraction.

2.2. Molecular tests

DNA extraction from maternal blood samples with and without RF was performed using the Illustra™ (Blood GenomicPrep Mini Spin - GE HealthCare – Little Chalfont, Buckinghamshire, UK) kit following the manufacturer's recommendations. To extract the genetic material from the foetal tissues, the GeneJet Genomic DNA Purification kit (Thermo Scientific) was used. The qPCR (quantitative PCR) tests were performed in the 7500 Fast (Applied Biosystems, Carlsbad, CA, USA) for detection of FeHV-1, FIV, FeLV, and CPPV-1 based on the methods of Vögtlin et al. (2002), Taniwaki and Araújo Jr. (2011), Tandon et al. (2005) and Decaro, Desario, Miccolupo et al. (2008b), respectively. PCR was performed on the qPCR samples that were positive for CPPV-1 and the VP2 gene based on the method of Buonavoglia et al. (2001) to obtain a larger amplification product of the positive sample for Sanger sequencing. Amplified PCR products were transferred to 1.5% agarose gel stained with Neotaq Brilliant Plus DNA stain at 0.5 µL/mL (NeoBio, SP, Brazil) and electrophoresis was performed. The agarose gel was observed under ultraviolet light, and the expected bands were purified and Sanger sequenced using Big Dye Terminator 3.0 (Applied Biosystem, Carlsbad, CA, USA). The sequences were analysed with MEGA 6.0 software and compared in the BLAST database. If maternal blood samples were positive for a pathogen, foetal tissues from the spleen and kidneys were tested by the previously described methods. All positive foetal and some maternal tissues were also sequenced.

2.3. Statistical analysis

A chi-square test was performed by cross-tabulation (non-parametric data) to evaluate the dependent variable, "animals with abortion and foetal death", in relation to the non-infectious variables obtained from the questionnaire (adapted from Lamm & Njaa, 2012). These included primiparous or multiparous queens, litter size and number of neonates, previous presentation of RF, contact with sick cats, contraceptive use, genetic tests, presence of ectoparasites, introduction of new animals, arrival of animals that had travelled, vaccination history and nutritional status. The statistical program SPSS version 22 was used.

An analysis of variance (ANOVA) between healthy animals (16 queens without RF) and RF animals (26 queens with RF) was performed to determine whether the groups differed in any of the parameters. A table containing the haematological data of the RF animals and the group of 16 healthy animals that came to the UFERSA HOVET was constructed. The variables used were red blood cells (RBC), haemoglobin, haematocrit, medium corpuscular volume (MCV), concentration of mean corpuscular haemoglobin (MCHC), leukocytes, segmented neutrophils, rods, eosinophils, lymphocytes, monocytes,

Table 1
Post mortem exam and molecular test results for the foetuses and queens with reproductive failure.

Queens	Macroscopy Uterus	Macroscopy Placenta	Macroscopy of foetuses	GA (days)	qPCR/PCR (CPPV-1)[a]	qPCR/PCR (CPPV-1)[b]
F1	N	N	FD	45	+	–
F2	H	H	FD	U	+[c]	–
F3	H	H	FD (Malformed skull)	49	+	–
F4	H	H	FD (Unformed eyes)	60	+	–
F5	N	N	FD	58	+	–
F6	H	H	FD	61	+	–
F7	H	H	FD (Malformed Abdomen and skull)	U	+	–
F8	N	H	FD (Malformed Abdomen)	U	+	–
F10	N	H	N	56	+	–
F11	H	N	N	53	+	–
F15	H	H	H	58	+	–
F16	H	H	FD	51	+	–
F17	H	–	NF	U	+	–
F18	N	H	H	51	+	–
F19	H	–	NF	U	+	–
F20	H	H	FD	47	+	–
F21	H	H	H	53	+	–
F22	H	H	H	U	+	–
F23	H	H	FD	50	+	–
F24	H	H	H	55	+	–
F25	N	H	H	U	+	–
F27	H	H	H	56	+	–
F28	H	H	FD	50	+	–
F29	H	H	FD	U	+[c]	+[c]
F30	H	H	FD	U	+[c]	–
F31	N	H	H	50	+	–

GA- Gestational Age;
[a] Blood of queens;
[b] Pool of foetal spleen/kidneys;
[c] Sample sequenced positive for feline panleukopenia; N- Normal; H- Haemorrhagic; FD- Foetal death/mummification; U- Undetermined; NF- No foetus (abortion at home, owner did not bring foetuses to the veterinary hospital).

platelets, ALT, AST, urea, creatinine, total protein, albumin, globulin and albumin/globulin ratio.

3. Results

None of the cats was vaccinated for FP, and none presented clinical signs related to the gastrointestinal tract such as enteritis, diarrhoea or vomiting. The possible non-infectious causes of infertility (primiparous or multiparous, size of litter and number of neonates, presence of cats who had already presented RF, contact with sick cats, contraceptive use, genetic tests, presence of ectoparasites, introduction of new animals, arrival of animals that had travelled, vaccination and nutritional status) were considered in the results evaluation but were statistically insignificant. From the macroscopic analyses, organs with haemorrhagic aspects and changes indicative of the mummification process are shown in Table 1.

The blood of queens without reproductive failures was negative for molecular investigations for the diseases studied. The 26 RF females were negative for FeHV-1, FIV and FeLV through qPCR. The same blood samples were tested by qPCR and PCR for CPPV-1, and all were positive (Table 1). After the all females with RF were positive for PCR/qPCR CPPV-1, three females were drawn (including the only mother with positive foetuses) for the sequencing to rule out cross-contamination and determine which CPPV-1 type is present in the samples. We proceeded to perform qPCR/PCR to verify CPPV-1 from the foetal spleen and kidneys. The three females tested and only the foetuses from sample F29 were positive, with an analysed sequence of 442 bp obtained by Sanger sequencing of the PCR product, which was 99% identical to the feline panleukopenia viral strain (CPPV-1 genus) sequences from GenBank HQ184193 (An et al., 2011); FJ440714; EU252145.

Statistical analysis of the haematological data revealed significant differences ($p < 0.05$) in the liver biochemistry (ALT and AST). Of the 26 RF females tested, 18 presented alterations in these enzymes, which were directly related to the foetal viability. In addition, 19 of the 42 females had significant changes ($p < 0.05$) in their relative segmented neutrophil counts, 22 had significant alterations ($p < 0.05$) in lymphocyte counts, both relative and absolute, and 18 had significant alterations ($p < 0.05$) in absolute monocyte counts (Table 2).

4. Discussion

In our study, no females (with and without RF) were positive for FHV-1, FIV or FeLV, a result that contradicted expectations. Per Givens and Marley (2008), viral agents are the most commonly reported causes of RF in felines.

Detection of viral nucleic acid is the gold standard for diagnosing CPPV-1 infection (Desario et al., 2005), with qPCR/sequencing being a useful tool in detecting both canine and feline infections (Decaro, Desario, Lucente et al., 2008a; Streck, Rüster, Truyen, & Homeier, 2013) because partial replication (of either field or vaccine CPV strains) can occur in the intestinal epithelium even in the presence of high titre serum and may interference with diagnostic assays (Decaro & Buonavlogia, 2017).

The molecular tests performed here were for CPPV-1, but cats may be infected with the CPV variant. However, CPV and other variants (CPV 2a or 2b) are known to infect cats, causing severe enteritis (Garcia et al., 2011; Lamm & Rezabek, 2008) and can be difficult to distinguish from the classic presentation of the FPV infection. One limitation of this study was that the PCR did not differentiate between the CPPV-1 types (all blood samples from the queens were CPPV-1-positive, suggesting a viremia) or determine the presence of coinfections. Coinfection is possible (Haynes & Holloway, 2012), however, our three females and one foetuses were positive for FP viral strain by Sanger sequencing. The coinfections studies evaluated cats with diarrhoea, and the present study evaluated queens whose only clinical sign was the presence of RF, suggesting only an FPV infection, as CPV does not cause reproductive symptoms in queens.

Table 2
Blood count/blood chemistry results for the queens with and without reproductive failure.

Queens	N	L	M	↑ALT	↑AST	Queens	N	L	M	↑ALT	↑AST
With reproductive failures[a]						**Without reproductive failures**					
F1	+	+	−	+	+	**F32**	+	−	−	−	−
F2	−	+	−	+	+	**F33**	+	+	+	−	−
F3	−	−	+	−	+	**F34**	+	+	−	−	−
F4	+	+	−	+	−	**F35**	+	+	+	−	−
F5	+	+	+	−	+	**F36**	+	−	−	−	−
F6	+	+	+	+	+	**F37**	−	−	−	−	+
F7	−	−	−	−	+	**F38**	+	−	+	+	−
F8	−	+	−	+	+	**F39**	−	+	−	−	−
F10	−	+	+	+	+	**F40**	−	−	+	−	−
F11	−	+	+	+	+	**F41**	−	−	−	+	−
F15	+	−	+	−	−	**F42**	−	−	−	+	−
F16	+	+	−	+	−	**F43**	+	−	+	−	−
F17	−	+	+	+	−	**F44**	−	−	−	−	−
F18	−	+	+	+	−	**F45**	+	−	+	+	−
F19	−	+	−	+	+	**F46**	−	−	+	−	+
F20	−	+	+	−	+	**F47**	−	−	−	+	−
F21	−	−	−	−	+						
F22	+	−	+	−	−						
F23	+	+	−	+	−						
F24	−	−	−	−	+						
F25	+	+	−	−	−						
F27	+	−	+	−	−						
F28	−	+	−	+	+						
F29	−	+	−	−	+						
F30	−	+	−	−	+						
F31	+	−	−	−	−						

N = Neutrophilia; L = Lymphopenia; M = Monocytosis; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase.
[a] Statistically significant ($p < 0.05$).

Per Truyen and Parrish (2013), CPV infection may include non-specific signs such as anorexia, fever, and diarrhoea, and in some cases, foetal or neonatal infections in bitches, reinforcing the possibility that the CPPV-1 found in the blood is the FPV due to the RF signs. About the females without RF, CPV infects felines (Decaro et al., 2012), establishes infection, and leads to either the development or the absence of clinical symptoms (Battilani, Bassani et al., 2006), however, the molecular tests were done to discard CPPV-1 and others reproductive diseases.

In Table 1, most of the foetuses are in the final stage of gestation, suggesting that the queens were chronically infected or undergoing repeated rounds of infection–clearance–reinfection. Although CPPV-1 typically causes acute infection, longer periods of infection have been reported (Clegg et al., 2012). In this study, abortion and natimortality were found, as well as mummified foetuses present in the uterus (Table 1). The FPV's action in the female reproductive system can develop several pathologies (Troy & Herron, 1986). Abortion, foetal mummification and stillbirth are commonly associated with infections that occur in the first one-third of the pregnancy (Lamm & Rezabek, 2008). We found that 10 animals (38%) showed those pathological abnormalities (mummification), which suggests that the queens were infected in the early one-third of their pregnancy (Table 1). Foetal brain damage and malformation indicate that the female was infected in the final one-third of gestation (Stuetzer & Hartmann, 2014). In this study, 4 queens had foetuses (15%) with these malformations, suggesting infection in the final gestational period.

The cats in this study did not have diarrhoea, which indicated FPV infection. As per the literature, the development of clinical symptoms depends on the satisfactory immune response, age and occurrence of possible secondary infections (Foley et al., 1999). The study by Haynes and Holloway (Haynes & Holloway, 2012) corroborates our results that the CPPV-1 may continue to proliferate in feline tissues without clinical signs. However, when the infected feline is pregnant, abortion is the only symptom that may occur (Decaro et al., 2012).

Because the mother cats' blood was positive for CPPV-1, some foetal tissues were tested for the virus, with negative results. However, with the PCR assay, one foetal tissue sample (from F29) was positive. This is explained by Givens and Marley (2008), who state that animals that develop pregnancies with multiple foetuses and are infected with a pathogen that can contaminate the foetus, the level of foetal infection and its consequences will differ individually. Although only the foetuses from one female (F29) were FPV-positive, all other foetuses died in utero or were aborted by the systemic action of FPV in the mothers, or were infected at the beginning of gestation, leading to mummification and consequently decreasing the chances of finding FPV DNA in foetuses. Per the literature, queens can experience abortion and RF due to the systemic action of the disease, severe debilitation and peracute presentation (Decaro et al., 2012; Greene, 2012; Verstegen et al., 2008).

CPV infections in cats may cause haematological abnormalities only (Chalmers et al., 1999); however, in our study, the queens also demonstrated RF. The result of the tests performed on the queens' blood samples was statistically significant for relative neutrophilia, lymphopenia and monocytosis, with foetal non-feasibility, demonstrating the FPV's role in reducing the WBCC. The presence of neutrophilia in the WBCC can occur in the late stages of FPV infection in cats (August, 1989) in which the leukopenia stage has already occurred or that have not yet developed leukopenia inherent to FP (Kruse, Unterer, Horlacher, Sauter-Louis, & Hartmann, 2010). Lymphopenia is associated with panleucopenia, which is common in FPV infections (Parrish, 1995). Monocytosis can occur in acute or chronic disease stages (Lopes, Biondo, & Santos, 2007) and may have occurred in the infections described in our study. The ALT and AST biochemistry results, when compared to the non-viable foetuses, were statistically significant. In a study with 244 cats with FP, 26.6% had altered AST and 13.3% had altered ALT (Kruse et al., 2010). The authors concluded that there was no correlation between clinical sign severity and the outcome of FP.

5. Conclusion

Current taxonomy defines CPPV-1 as one single taxonomic entity, with five species of viral strains, including CPV and FPV (Cotmore et al.,

2013). CPV is responsible for enteric disease in cats and dogs, infecting many tissues and causing many diseases, including foetal death after systemic infection in foetuses (Garcia et al., 2011; Truyen & Parrish, 2013). Studies show that FP is caused by classic FPV strains (Decaro, Desario, Miccolupo et al., 2008b). In Brazil, feline faecal samples were confirmed to contain FPV by nucleotide sequencing (Garcia et al., 2011). Our results found that FPV has the potential to alter the disease in cats, as the queens demonstrated only clinical signs of RF associated with WBCC abnormalities. Evidence of protoparvoviral infection has been found in post-mortem feline tissues, suggesting that these viruses may contribute to clinical syndromes other than panleucopenia and cerebellar degeneration in cats (Url, Truyen, Rebel-Bauder, Weissenbock, & Schmidt, 2003). The tissues in this study did not contain the viral agents commonly reported to cause RF in domestic queens: FIV, FeLV, and FHV-1. Furthermore, FPV was found in queens without classical FP gastrointestinal signs. Therefore, we can exclude FHV-1, FIV and FeLV as causing the reproductive pathologies in this study, as our findings suggest that FPV is their main cause. There are previous reports of RF in animals who were infected with this virus, giving scientific support to our results. We found no reports in the literature that indicated CPV as a cause of RF in queens. The animals experienced abortion or foetal death due to viral systemic action or by the direct action of FPV on maternal or foetal reproductive tissue. These results support the hypothesis that FPV caused the RF reported in this study and was associated with significant WBCC alterations.

Conflicts of interests

The authors declare that are no conflicts of interests.

Acknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2018.06.004.

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