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INNOVATIVE APPROACHES
FOR THE CLINICO-PATHOLOGICAL DIAGNOSIS
OF FELINE INFECTIOUS PERITONITIS
AND FELINE CORONAVIRUS INFECTION

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Abstract

Feline infectious peritonitis (FIP) is a deadly disease of felids with a viral and immune-mediated pathogenesis. The nature of the etiological agent – feline coronavirus, FCoV – and the non specific clinical presentation make this disease particularly challenging both from a pathogenetic and a diagnostic point of view. Many aspects still represent an issue, like not knowing the mutation(s) responsible for the development of FIP, the lack of a gold standard for the diagnosis of FIP *in vivo* and the absence of an effective treatment. This thesis was aimed to clarify some of these aspects, specifically: a novel test on effusions was developed (namely, Δ total nucleated cell count – Δ TNC – i.e. the ratio between the two white blood cell count provided by the Sysmex XT-2000iV analyzer) (studies I and II); the frequency of atypical serum protein electrophoresis (SPE) patterns in cats with FIP, anecdotally reported during our diagnostic activity (study III) was investigated, a comparison of clinico-pathological and molecular tests for the diagnosis of FIP (study IV) was performed, a loop isothermal amplification method (LAMP) for the detection of FCoV was developed (study V) and an investigation on the prevalence of two mutations of the spike (S) protein gene in a wide number of samples from FIP and non-FIP cats was carried out (this latter study developed during an externship at the University of Bristol in collaboration with Prof. Séverine Tasker and Dr. Emi Barker) (study VI). The results of studies I and II demonstrated that the Δ TNC is a reliable method to support the diagnosis of FIP either on fresh or on frozen effusions. Study III confirmed that SPE profiles consistent with FIP are less frequent in recent years than in the past, possibly due to changes in the pathogenic characteristics of the FCoVs. However, study IV demonstrated that: on blood molecular tests may support a clinical diagnosis of FIP but none of the test, except the measurement of α_1 acid-glycoprotein (AGP) may rule out this disease; cytology should be preferred on effusions either to exclude or confirm the disease and, on tissues, S gene sequencing should be preferred when histology is highly consistent with FIP while 3' UTR PCR when FIP is less likely; the LAMP method developed in study V may be used to confirm the presence of FCoVs in the samples but is poorly sensitive and cannot exclude the presence of FCoVs. Finally, pyrosequencing of FCoV performed in study VI demonstrated the presence of gene S mutations also in FCoVs from fecal samples. The analysis of sequences recorded in this latter study, however, is still ongoing and future results may provide new insights on the pathogenesis and diagnosis of FIP.

Abstract (italian version)

La peritonite infettiva felina (FIP) è una patologia letale dei felidi, a patogenesi virale ed immunomediata. La propensione dell'agente eziologico alle mutazioni – coronavirus felino, FCoV – e la sintomatologia spesso aspecifica, rendono questa patologia complessa sia dal punto di vista diagnostico che patogenetico. Non si è ancora a conoscenza, infatti, della mutazione virale responsabile della patologia, non esiste ancora un gold standard per la diagnosi *intra vitam* e non è ancora disponibile una terapia valida. Lo scopo di questa tesi è di tentare di chiarire alcuni di questi aspetti. I primi due obiettivi (studio I e II) erano diretti allo sviluppo di un test per la diagnosi di FIP in forma effusiva. In particolare, è stata valutata l'accuratezza diagnostica del valore ottenuto dal rapporto tra le due conte leucocitarie fornite dal Sysmex XT-2000iV (Δ total nucleated cell count – TNCC) su campioni di versamento FIP indotto. Successivamente, è stata valutata la possibilità di effettuare lo stesso test su surnatanti di versamenti dopo aggiunta di sangue intero felino, in modo da poter ottenere questo valore su campioni congelati o con risultati dubbi ad altri esami. Il terzo scopo (studio III) era volto a confermare o smentire la presenza di pattern elettroforetici atipici in corso di FIP, come registrato nel nostro laboratorio negli ultimi anni. È stato quindi svolto uno studio retrospettivo per confrontare pattern elettroforetici in due periodi di tempo diversi. Il quarto obiettivo (studio IV) si prefissava di valutare l'accuratezza diagnostica di diversi test, sia clinico patologici che molecolari, per trovare il miglior test o la miglior combinazione di test per la diagnosi di FIP in vivo. Nello stesso studio è stato anche valutato il sequenziamento del gene spike (S), ultimamente proposto come discriminante, quando mutato, tra i due patotipi del FCoV. Il quinto scopo (studio V) era di mettere a punto una metodica molecolare isotermica (loop isothermal amplification method – LAMP) per il rilevamento del FCoV. Questa metodica, essendo veloce ed economica, potrebbe facilitare l'identificazione dei gatti eliminatori del FCoV o, per alcuni campioni, la diagnosi di FIP. Durante il mio percorso di dottorato ho anche partecipato ad un progetto sotto la supervisione della prof. Séverine Tasker e della dott.ssa Emi Barker dell'università di Bristol. Questo progetto (studi VI e VI.1) ha lo scopo di scoprire la vera prevalenza, in un ampio numero di tessuti, fluidi e feci ottenute da gatti affetti e non affetti da FIP, di due mutazioni del gene spike considerate ultimamente come co-responsabili della FIP.

Gli studi I e II hanno dimostrato che il Δ TNC può essere usato per diagnosticare la FIP con buona accuratezza. Lo studio III ha confermato che i pattern elettroforetici tipici di FIP sono meno

frequenti negli ultimi anni, possibilmente per modificazioni nella patogenicità dei FCoV. Dallo studio IV si evince che i test molecolari possono confermare la diagnosi di FIP, ma che solo l'AGP può escluderla; l'esame citologico dei versamenti dovrebbe essere il test di scelta sui versamenti, mentre sui tessuti il sequenziamento del gene S dovrebbe essere usato per confermare la diagnosi, mentre la PCR 3' UTR PCR quando la FIP è meno probabile. La metodica LAMP sviluppata nello studio V si è rivelata molto specifica ma poco sensibile, dimostrandosi un buon test per confermare la presenza di FCoV in campioni biologici, ma non per escluderla. Infine, lo studio VI ha messo in evidenza la presenza di coronavirus mutati anche nelle feci di gatti non affetti da FIP, mostrando che i successivi progressi in questo studio metteranno in evidenza nuovi aspetti della patogenesi della FIP.

List of Publications

The works presented in this thesis are based on the following publications:

- I. Giordano, A., Stranieri, A., Rossi, G., Paltrinieri, S., 2015. High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis. *Veterinary Clinical Pathology*, 44(2): 295-302.
- II. Stranieri, A., Paltrinieri, S., Giordano, A., 2016. Diagnosing feline infectious peritonitis using the Sysmex XT-2000iV based on frozen supernatants from cavitory effusions. *Journal of Veterinary Diagnostic Investigation*, E-pub ahead of print. doi: 10.1177/1040638716689115
- III. Stranieri, A., Giordano, A., Bo, S., Braghiroli, C., Paltrinieri, S., 2016. Frequency of electrophoretic changes consistent with feline infectious peritonitis in two different time periods (2004-2009 vs 2013-2014). *Journal of Feline Medicine and Surgery*, E-pub ahead of print. doi: 10.1177/1098612X16664389.
- IV. Stranieri A., Giordano, A., Paltrinieri, S., Giudice, C., Cannito, V., Lauzi, S., 2016. Comparison of the diagnostic performances of laboratory tests for the diagnosis of feline infectious peritonitis. *Veterinary Record* [under review].
- V. Stranieri, A., Lauzi, S., Giordano, A., Paltrinieri, S., 2016. Reverse transcriptase loop-mediated isothermal amplification for the detection of feline coronavirus. *Journal of Virological Methods*, 243: 105-108. [E-pub ahead of print].

Angelica Stranieri also participated to a project led by Prof. Séverine Tasker and Dr. Emi Barker of the University of Bristol.

- VI. Feline coronavirus spike protein in cats with and without feline infectious peritonitis. Preliminary results were presented at the fourth international meeting of the International Society for Companion Animal Infectious Diseases (ISCAID) with the abstract:
 - VI.1. Barker, E.N., Stranieri, A., Helps, C.R., Porter, E., Davidson, A.D., Day, M.J., Kipar, A., Tasker, S., 2016. Feline coronavirus shedding in cats with and without feline infectious peritonitis.

The contribution of Angelica Stranieri to the listed papers and projects are the following:

- I. Took minor part in the laboratory work, had the main responsibility of writing the paper after revision.
- II. Took major part in study design, performed most of the laboratory work (samples collection and test optimization), interpreted the results together with supervisor and co-authors and had the main responsibility of writing the paper.
- III. Took major part in the study design and in the retrospective analysis of data, interpreted the results together with supervisor and co-authors, had major part in writing the paper.
- IV. Took major part in the study design, performed most of the necropsies and sample collection, RNA extraction and clinic-pathological analyses. Regarding *post mortem* analyses, the candidate prepared the samples for paraffin embedding, assisted the technician for the microtomic sections preparations and performed immunohistochemistry with the supervision of co-authors. The candidate also interpreted the results together with supervisor and co-authors and had major part in writing the paper.
- V. Took major part in the study design (primers and work conditions design) and in the laboratory work (RNA extraction, gel preparation and LAMP experiment with co-authors); interpreted the results together with supervisor and co-authors and had major part in writing the paper.
- VI. Took major part in the sample collection and processing and in the RNA extraction. Took minor part in the PCR and pyrosequencing and interpreted the results with the supervisors.

SCIENTIFIC BACKGROUND

Introduction

It was 1963 when the soon to be known feline infectious peritonitis (FIP), a world-wide spread, fatal disease of felids, appeared in the scientific milieu as one “important disorder of cats” (Holzworth, 1963). Even if the etiological agent of this disease was at that time unknown, some of the characteristics described by the Author are still actual, like the frequent occurrence of FIP in young cats and the sadly actual lack of any effective treatment (Holzworth, 1963; Pedersen, 2009). In the following years, a viral etiology was first proposed by Wolfe and Griesemer (1966) and later experimentally confirmed and ascribed to the feline coronavirus (Zook et al., 1968; Ward, 1970; Horzinek et al., 1977; Pedersen et al., 1978). Feline Infectious Peritonitis (FIP) is believed to be a major cause of infectious disease-associated deaths in the feline population (Hartmann, 2005). FIP was and still is an extensively studied disease but, despite the enormous progresses made, several aspects about both the pathogenesis and the diagnosis of FIP are still yet to be known, making FIP one of the most feared as well as challenging disease of felids (Kipar and Meli, 2014; Pedersen, 2014a).

Feline coronavirus

Coronaviruses are well known for their impact on both human and animal health, being the causative agents of diseases like severe acute respiratory syndrome (SARS), middle eastern respiratory syndrome (MERS), infectious bronchitis in chickens and transmissible gastroenteritis in pigs (Weiss and Navas-Martin, 2005; Hilgenfeld and Peiris, 2013). Feline coronaviruses (family *Coronaviridae*, order *Nidovirales*) are enveloped, single-stranded positive sense RNA viruses belonging to the species *Alphacoronavirus 1*, genus *Alphacoronavirus* of the sub family *Coronavirinae* (Gonzales et al., 2003). Feline coronavirus genome is about 30 kb long and organized in 11 open reading frames (ORFs) (Figure 1). The majority of the genome is represented by the two 5' proximal, overlapping ORF 1a and ORF 1b, which are translated as polyproteins pp1ab and pp1a by frame-shifting and non frame-shifting mechanisms respectively (Sawicki et al., 2005; Pedersen, 2014a). These polyproteins include 16 non-structural proteins (nsps) which have replication associated activities (genome replication, proteolytic processing and multiple subgenomic mRNA synthesis) and form a membrane-bound replication-transcription complex through interaction with the endoplasmic reticulum and Golgi apparatus of the infected cell (Prentice et al., 2004). The remaining 9 ORFs encode for four structural proteins (envelope, E; membrane, M; nucleocapsid, N and spike, S) and for 5 group-specific, accessory proteins (3a-c, located between the S and the E genes, and 7a-b, located at the 3' end of the genome) (Haijema et al., 2004).

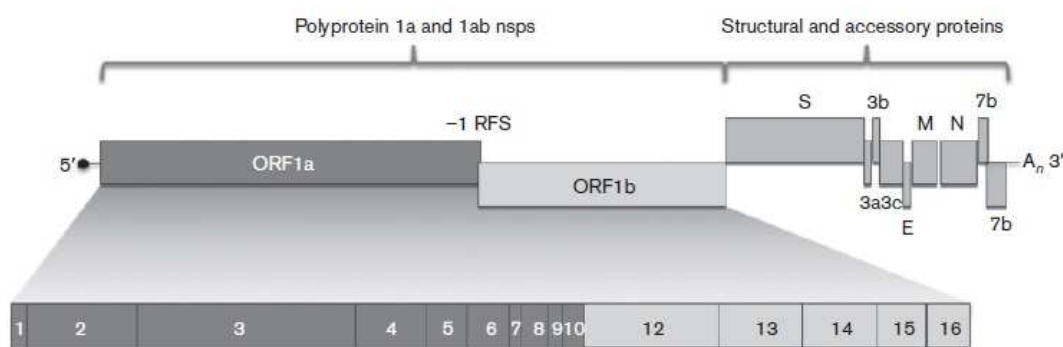


Figure 1. FCoV genome organization. From 1-16 the non structural proteins translated by the ORF1a and 1b are represented (Lewis et al., 2015).

These proteins are expressed from a set of 3' coterminal subgenomic (sg) mRNAs generated via discontinuous transcription during subgenome-length minus-strand RNA synthesis (Hagemeijer et al., 2012). The E, M and S proteins constitute the viral envelope, which surrounds the viral RNA

genome in association with the nucleocapsid protein (Li, 2016). The **Envelope (E)** proteins are small (around 9 kDa) hydrophobic, non glycosylated viroporins which mainly distribute between the endoplasmic reticulum and the Golgi apparatus membranes of the infected cell, where they actively participate in virion assembly, morphogenesis and egress (Dye and Siddell, 2005; Venkatagopalan et al., 2015). The **membrane (M)** proteins, despite their low molecular weight (around 30 kDa), are the most abundant protein in the viral envelope, with three transmembrane (TM) domains and a conserved overall structure among coronaviruses. Together with the E proteins, they play important roles in the virions morphogenesis, assembly and budding (Dye and Siddell, 2005; Ujike and Taguchi, 2015). The **nucleocapsid (N)** protein has a molecular weight of around 43 kDa and its primal role is to package the viral RNA in into long, flexible, helical complexes called nucleocapsids, which are incorporated during the budding process of the viral infection. It is dynamically associated with the replication-transcription complex and it also plays an essential role in the viral genome replication and in the sub genomic RNA synthesis (Dye and Siddell, 2005; Gorbalenya et al., 2006; Verheije et al., 2010; McBride et al., 2014). The N protein is the most immune-dominant FCoV antigen and several studies demonstrated its potential role in the protection against FIP, being responsible of a cell-mediated immune response (Hodatsu et al., 2003; Battilani et al., 2010; Rossi et al., 2011). The **spike (S)** proteins (160 kDa) are responsible for the name coronavirus (*corona* is the latin word for crown), since they form 20 nm long protrusions that extend from the viral envelope (Bosch et al., 2003; Dye and Siddell, 2005; Li, 2016). They are class I virus fusion proteins formed by three segments: a large ectodomain composed by a receptor-binding subunit (S1) and by a membrane-fusion subunit (S2), a single-pass transmembrane anchor, and a short intracellular tail. The S1 subunit is responsible of binding to receptors on the host cell surface for the virus entry, while the S2 subunit allows the viral genome to enter the host cell, through fusion of the host and viral membranes (Li, 2016). Coronavirus accessory proteins are thought to be not essential for *in vitro* replication, but to play essential roles for virus-host interactions, viral virulence and pathogenesis. To this day, feline coronavirus accessory proteins exact roles are still unclear (Dedeurwaerder et al., 2013; Liu et al., 2014). The **3a** and **3b** accessory proteins consist of 71 amino acids (aa) and they probably have intracellular functions, due to the lack of signal peptides or transmembrane domains. The **3c** protein is a class III triplemembrane spanning protein of 238-244 aa, resembling the M protein of the FCoV and the 3a protein of the SARS-CoV (Dedeurwaerder et al., 2013). This protein seems to be necessary for the survival and replication of the virus in the enteric environment (Chang et al., 2010). The **7a**

protein is a hydrophobic, well conserved among FCoV proteins of 101 AA. The **7b** protein consists of 207 aa and has shown several single amino acid polymorphisms among FCoV strains. It is secreted from infected cells and it is probably involved in the modulation of the host immune response (Herrewegh et al., 1995a; Dedeurwaerder et al., 2013). 7a and 7b proteins appear to be essential for an efficient replication in vitro as well as for virulence in vivo (Haijema et al., 2004; Dedeurwaerder et al., 2013). It was also recently shown that the 7a protein could protect the virus survival acting as a type I interferon (IFN) antagonist, but it can exert its antagonistic functions only through the presence of the 3a-c proteins (Dedeurwaerder et al., 2014).

After the S protein-mediated recognition and binding of the virus to the host cell, the coronavirus envelope must fuse with the target cell membrane in order to release the viral genome in the host cell cytoplasm (Cavanagh, 2005). This process seems to be mediated by cleavage of the S1/S1 subunits that, in feline coronaviruses, is performed by specific monocytes/macrophages proteases not yet fully characterized (Licitra et al., 2013). After the entry in the host cell, coronaviruses induce the formation of membranous structures called double membrane vesicles (DMVs), described only for SARS-CoV and mouse hepatitis virus (MHV) and in a not still completely understood manner, on which the replication transcription complex is anchored and the RNA synthesis occurs (van Hemert et al., 2008; Perlman and Netland, 2009; Hagemeijer et al., 2012). The replication takes place through RNA-dependent RNA polymerase (RdRp). The genomic positive-sense RNA is copied into a negative-sense template until it reaches a transcription-regulation sequence (TRS). At this point, RNA synthesis may either continue or the negative-sense sgRNA is completed after the relocation of the RdRp to the 5' end of the genome. The synthesized RNA is then incorporated into the virions on membranes located between the endoplasmic reticulum (ER) and the Golgi apparatus (ER-Golgi intermediated compartment: ERGIC) (Hagemeijer et al., 2012). After the translocation of the RNA and the N protein to the ERGIC and the Golgi region, where the viral glycoproteins are located, the newly formed virion is released from the host cell through the budding process (Stertz et al., 2007).

Among the RNA viruses, coronaviruses possess the largest genome and they lack of a proofreading activity, making mutations easy to occur. Even if mechanisms of “alternative” proofreading have been experimentally demonstrated (the inactivation of the nsp14, which encodes for an exonuclease, of SARS-CoV and MHV leads to a 15- to 20-fold increased mutation rates), mutations do happen and induce some of the most important characteristic of RNA viruses: the ability to adapt themselves to different environments and to change cell or even host tropism. The result of

this process is the formation of quasispecies, which are related but not identical genomes, subjected to not stopping processes of genetic variations, competition and selection (Denison et al., 2011; Domingo et al., 2012). The concepts of quasispecies formation and genetic recombination typical of coronaviruses are important in order to understand feline coronaviruses behavior and their subdivision in two serotypes (FCoV I and FCoV II) as well as in two pathotypes (feline enteric coronavirus, FECV; feline infectious peritonitis virus, FIPV). The two FCoV serotypes can, in fact, be recognized based on their link with canine coronavirus (CCoV). Virus-neutralizing antibody reactions and comparison of amino acid sequences of the spike protein demonstrated that the FCoV II is the result of a genome recombination between the CCoV and the FCoV, where the CCoV spike gene was incorporated into FCoV type I (Herrewegh et al., 1995a; Motokawa et al., 1996). Type I FCoV grows poorly in vitro, while type II strains grow well and are, for this reason, more used for experimental studies. This is in contrast with what happens in the field, since type I FCoVs are more globally diffused, while type II FCoVs are less diffused and represent the 10-20% of FCoVs isolated in Asia (Rottier, 1999; Sharif, et al., 2010; Pedersen, 2014a). The two strains also differ for the receptor used to enter the cells. Feline aminopeptidase N is as a receptor for type II FCoV, but not for type I FCoV. Type I FCoV uses a feline dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (fDC-SIGN) as a co-receptor, together with a mannose-binding lectin or another receptor of still unknown origins (Regan et al., 2010; van Hamme et al., 2011).

Feline infectious peritonitis

Role of FCoV mutations

The two pathotypes of FCoV refer to their capability of causing FIP. This because while it is now fully accepted the hypothesis for which FIPV derives from mutations of the FECV within each cat, it is still not understood which is the real mutation responsible for this switch. As reported by Gunn-Moore et al. (1999): “FCoV exists within individual cats as complex viral populations”. When one of the quasispecies generated during the replication-transcription process acquires the capability of changing cell tropism from enterocytes to monocytes/macrophages, along with an impaired immune response of the host, FIP occurs (Pedersen, 2009). FECV, in fact, is primarily confined to the intestinal tract, where it causes a mild enteritis (Rottier, 1999). The FECV was believed for years to be confined to the host intestinal epithelium, but several studies have demonstrated that FECV can spread systematically through monocytes-associated viremia in infected, but healthy, cats (Gunn-Moore et al., 1998a). Nevertheless, the FECV was also demonstrated not only to be carried, but also to freely replicate in the host monocytes, when this was believed to be only a FIPV trait. Moreover, cats shed FCoV in the feces after been inoculated intraperitoneally with the FECV, even if to a lesser extent compared with cats inoculated with FIPVs. Thus, the real difference between the two pathotypes seems to be the higher and more effective rate of replication inside the blood monocytes of the FIPV compared with the FECV (Simons et al., 2005; Can-Sanha et al., 2007; Pedersen et al., 2012).

The first gene taken into account for its role in the FECV-to-FIPV switch was the accessory 3c gene. The majority of the FIPVs isolated from FIP cats showed deletions in the 3c gene (Vennema et al., 1998). Structural and accessory genes of FIPV strains obtained from affected tissues were sequenced, showing that the highest number of mutations were located in the 3c gene and were represented mostly by randomly scattered single nucleotide polymorphisms (SNPs) that led to variable truncations of the 3c protein. These mutations were also present in the FECVs obtained from the feces of the same cats, but to a lesser extent. Interestingly, the type and number of nucleotide deletions were always different between the different strains, confirming that each mutation arises independently in each cat (Pedersen et al., 2009). When the 3c genes were sequenced from a higher number of FIPVs and FECVs, mutations in the 3c gene were found in the majority, but not in all, the FIPVs, while none of the FECVs obtained from feces showed the mutations. The authors concluded that an intact 3c gene is essential for the replication in the host

intestine, but also that 3c gene mutations are not the only responsible for the FECV-to-FIPV switch (Chang et al., 2010). This latter finding was later on confirmed, since FIPVs with mutated 3c genes were not shed in the feces after oronasal inoculation, while FIPVs with an intact 3c gene were. Again, the 3c gene mutation was confirmed not to be the only cause of FIP, since both mutated and non mutated 3c gene FIPVs were capable of inducing FIP after oronasal or intraperitoneal inoculation. Additionally, FIPVs with an intact 3c gene contained more amino acidic changes in the 3c protein than FECVs, making this gene very variable between different strains (Pedersen et al., 2012). A more recent study investigated the clonal diversity of 3c genes obtained from FIP and non FIP cats, with the aim to detect not only the dominant sequences but a higher number of sequences that may harbor the mutation. As a result, all the FIPVs showed the truncated 3c gene, in contrast with what previously reported and consequently defining the 3c gene as a promising marker for the FECV-to-FIPV switch (Hora et al., 2016). The real function of the 3c gene is, however, still not clear. Due to its hydropathy profile similar to that of the SARS-CoV 3a protein, it was postulated that the 3c protein could have similar functions (pro-apoptotic properties and virion release), but this still remains only a hypothesis (Pedersen, 2014a). A recent study showed that the 3c protein could have a virulence suppressing effect, aimed to maintain the symbiosis between the host and the virus, using a pathway not involved with autophagy. It was also hypothesized that a truncated 3c protein could subsequently enhance the viral replication in macrophage-like cells (Hsieh et al., 2013).

The ORF 7a/7b were also early investigated as potential markers of virulence. A first study by Herrewegh et al. (1995a) showed that while the 7a protein was well conserved among FCoV strains, the 7b protein amino acid sequences were less similar between strains. Moreover, when comparing avirulent FIPVs strains with FCoVs 7b sequences after tissue culture passages, nucleotide deletions of different lengths were found in the avirulent FIPVs strains. When the same comparison was performed on samples collected from naturally infected and FIP affected cats, the deletion in the ORF 7b were not present. The authors concluded that while the ORF7b is not necessary for *in vitro* replication, thus easily lost, it somehow gives advantages for *in vivo* replication and that deletions in this protein could diminish the virus virulence (Herrewegh et al., 1995a). The same findings were confirmed by another study, where viruses with a truncated 7b gene have been associated with enteric coronaviruses only (Vennema et al., 1998), but when the expression of the 7b protein was investigated as its mutation could really be a distinctive tract between FECV and FIPV, the results were quite different. A study aimed to evaluate the antibody

response against the 7b protein in FIP affected cats demonstrated that both FIP, non FIP and healthy cats had antibodies against this protein (Kennedy et al., 2008). In a following study, 7b proteins with deletions were found both in FCoV from fecal samples of infected, healthy cats and in effusions of FIP affected cats. Thus, the presence of deletions in 7b protein is not correlated with the viral pathogenicity and cannot be used to distinguish FECVs from FIPVs (Lin et al., 2009). The role of the spike gene in the pathogenesis of FIP has began to be thoroughly investigated in the last few years, precisely after a paper published in 2012 by Chang et al. In this study, 11 FCoVs obtained from tissues of FIP affected cats and 11 FCoVs obtained from feces of healthy cats were sequenced and subjected to a screening for nucleotide differences among the two pathotypes. In the 10% of the genome positions, the nucleotide identity in at least 1 of the 11 FIPVs did not occur in any of the 11 FECVs and almost a half of these differences were due to differences in the spike gene. One nucleotide difference was found in 9/11 FIPVs (nucleotide T or C at position 23531) and in none of the FECVs (nucleotide A at position 23531). The mutation of this single nucleotide leads to the modification of one amino acid (from methionine to leucine at position 1058) in the fusion peptide of the spike protein. When analyzed on a larger scale, the same finding was present in the 96% of FIP cats, while another single nucleotide mutation in close proximity (mutation S1060A) allowed to additionally distinguish FIPVs from FECVs. Given the functions of the spike protein in coronaviruses (cell targeting and cell entry) the authors concluded that this mutation is probably associated with the FIPV pathogenicity, but that is also most likely not the only responsible for the FECV-FIPV switch (Chang et al., 2012). A year later, a study from Licitra et al. (2013) showed a set of at least one nucleotide mutations in the S1/S2 furin cleavage site of FIPVs, but not in the FECVs. In particular, more than 40 % of FIPVs showed mutations (e.g. deletions) in the arginine residue of the P1 position of the cleavage site. The next more common mutations of the FIPVs was in the P2 position. These mutations, however, even if believed to be associated with an enhanced tropism for monocytes and macrophages, were found only in two thirds of the genomes analyzed and led also to a sometimes higher, sometimes lower cleavage efficiency, leaving doubts about the real meaning of these findings (Licitra et al., 2013). The same author extended this work by additionally investigating possible mutations at the S2' cleavage site, which is the second cleavage activation site of the S protein, from FECVs obtained from feces of healthy cats and from FIPVs collected by FIP clinically and sometimes immunohistochemically diagnosed cats. Again, mutations were present in both the cleavage sites obtained from FIPVs, but the patterns were complex and often

different between the different cats or even samples from the same cat, both in terms of interested position and in terms of amino acid change in the same position (Licitra et al., 2014). In a more recent study, the main mutation described by Chang et al. was investigated in an extended cohort of fecal and tissue samples from both FIP and non FIP cats. Surprisingly, 9% of the tissue from FIP cats did not showed the mutations proposed by Chang et al. (2012) and, at the same time, 89% of tissues from non FIP cats showed the mutation M1058L described by Chang as typical of FIP. This result can be explained with a possible association of the mutation with a systematic spread of the FCoV, rather than to a pathogenic role of the mutated gene (Porter et al., 2014).

Epidemiology, shedding and transmission

Even if with wide variability among different countries, feline coronavirus is worldwide spread as well as feline infectious peritonitis (Drechsler et al., 2011). The prevalence of FCoV is extremely high, especially in multicat environments, where the seroprevalence can reach more than 90%, while in single-cat households it is between 10-50% (Horzinek and Osterhaus, 1979; Addie and Jarrett, 1992; Addie et al., 2000; Pedersen et al., 2004). Feline coronavirus is fecally-orally transmitted, therefore the risk of becoming infected increases in crowded conditions (Cave et al., 2004). Due to its high prevalence, FIP could be expected to be a very common disease. Conversely, its incidence is low, and only 5-12% of FCoV infected cats develop FIP (Drechsler et al., 2011). The viral fecal shedding usually begins a week after experimental infection with FECVs or at 9-10 weeks of age for kittens born from infected queens, and continues for up to 10 months, after which the pattern of shedding can follow three ways. About 5% of the cats develop a strong immune response and stop the shedding after an average time of 12 months; about 70-80% of the cats discontinuously shed the virus (intermittent shedders) and 10% of the cats persistently shed the virus for more than 24 months and possibly lifelong (Foley et al., 1997; Addie and Jarrett, 2001; Pedersen et al., 2008). The persistent shedders seem to be persistently infected with the same strain of the virus, while cats that recover from the infection are susceptible to be re-infected, either with the same or different viral strains (Addie et al., 2003). Persistent, healthy shedders, with the colon as the main site of viral persistence, play a key role in the epidemiology of FIP (Meli et al., 2004; Kipar et al., 2010).

Viral shedding is also correlated with the antibody titer, since cats shedding the virus usually have higher titers in comparison with non shedders (Pedersen et al., 2008). There are several risk

factors for the development of FIP. FIP usually occurs in young cats, typically after 5-6 weeks of age, when the maternal antibodies decrease. Stressful events (e.g. surgery, moving, co-infections) can be risk factors, as well as the proportion of shedders in the cattery, since the entrance of FCoV infected cats in multi-cat environments can increase the viral shedding from 10- to 1 million-fold in one week only. It has been in fact postulated that higher levels of viral replication could lead to the occurrence virulent mutants among the quasispecies generated by infected animals due to the high rate of viral mutations (Foley et al., 1997; Gunn-Moore et al., 1999; Hartmann, 2005).

Moreover, FIP seems to occur more often in male, intact cats and during fall-winter, probably due to the fact that cats tend to share the same environments (Rohrbach et al., 2001; Pedersen et al., 2004). Certain breeds are reported to be more likely to develop FIP: Birman, Ragdoll, Rex, Abyssinian and Himalayan breeds (Pesteanu-Somogyi et al., 2006).

FIP usually arises as an enzootic disease, with rare and unpredictable losses, usually of 1-5% distributed in a 5 year or longer period. Occasionally, however, it can arise as an epizootic disease, with typical FIP outbreaks characterized by the occurrence of FIP in about 10% of the cats, depending on the general prevalence of FIP in the given environment. The causes of FIP outbreaks are multifactorial and due to overcrowding, increased intestinal replication rate and a general increased stress level (Pedersen, 2009; Drechsler et al., 2011). Outbreaks events, even if rare, could suggest the presence of virulent strains capable of inducing FIP in a given population (Brown et al., 2009). This hypothesis was recently proved wrong, when it was demonstrated that the viral strains isolated during an FIP outbreak from FIP and FCoV infected cats were very closely related and that no evidence of circulating virulent-avirulent strains was found (Barker et al., 2013).

Pathogenesis of FIP

There are several unclear aspects about the pathogenesis of FIP. As stated above, it is still unknown, for example, the mutation responsible for the FECV-to-FIPV switch. Several studies published in the last years allow to recognize some key factors in the pathogenesis of FIP, which are the change of tropism of the FCoV from enterocytes to monocytes, the acquired capability of actively replicating in these cells, and an imbalance between the host cell-mediated and humoral immune response (Kipar and Meli, 2014). After oral experimental infection, FCoV targets the intestinal columnar epithelial cells. It is then probably taken up by enteric macrophages to the lymph node, from where they can be systemically spread and detected in the blood as well as in

the tissues resident macrophages (Kipar et al., 2010). The site of FECV-to-FIPV switch is still unknown, but the blood monocytes could intermediate this process (Pedersen, 2014a). The manifestation of FIP and how it clinically manifests depend on the host immune response. FCoV infected cats develop antibodies against the virus, no matter on the following course of the infection but, while some animals clear the infection, many remain infected or develop FIP (Vogel et al., 2010). The humoral response has, in fact, a supportive role in the development of FIP, while a strong cellular immune response has a protective role against the disease (Pedersen and Black, 1983). Cats with FIP show a decreased Th1 protective response, with a general decrease in the lymphocyte number and a specific decrease in the T cell subsets, which occurs very early after experimental infection and correlates with viral replication in blood (Gunn-Moore et al., 1998b; Kipar et al., 1998; Kipar et al., 2001; Paltrinieri et al., 2003; De Groot-Mijnes et al., 2005). The cytokine patterns found in FIP cats support these findings, since an inhibition of the cellular immune response due to the lack of the interleukin 12 (IL-12) leads to activation of monocytes and macrophages and, eventually, to FIP. On the other hand, FCoV-infected, healthy cats can avoid this hyperactivation thanks to the up-regulation of the interleukin 10 (IL-10), a cytokine with anti-inflammatory activities (Kipar et al., 2006a). Moreover, an upregulation of the tumor necrosis factor α (TNF- α) and a decrease in the interferon γ (IFN- γ) are generally found in FIP cats, since these two cytokines have an inducive and a protective role in the development of FIP, respectively (Kiss et al., 2004; Gelain et al., 2006). TNF- α , in fact, could be responsible for both induction of apoptosis in T cells, primarily CD8⁺ T cells, and for the increased expression of feline aminopeptidase N, the receptor used by FIPV II for entering the monocytes (Takano et al., 2007a; Takano et al., 2007b). In other studies, similar concentrations of IFN- γ were found in the blood of FIP and healthy cats, while high level were found both in FIP effusions and lesions. It is therefore more likely that this cytokine is released at the lesion level, where it increases macrophages hyperactivation and viral uptake (Berg et al., 2005; Giordano and Paltrinieri, 2009). Humoral response has been shown to support FIP development through a antibody-mediated enhancement (ADE) process. Antibodies against the spike protein facilitate the uptake of the virus from the monocytes through Fc receptors. This finding, however, seems to be more evident *in vitro* than *in vivo* (Olsen et al., 1992; Addie et al., 1995). If ADE plays only a secondary role in the infection *in vivo*, the question why the infected monocytes are not able to clear the infection remains open. A recent study investigated the role of the innate immune response, showing that FIP affected cats have a drastic decrease in the natural killers (NK) and T regulatory (Tregs) cells in

blood, lymph nodes and spleen. NK have anti-viral activities, while Tregs modulate the immune response and their decrease could explain the incapability of inducing both a decrease of the viral load and the monocytes activation. Curiously, intralesional NKs do not change in number and show activation, but this finding does not translate in an increased cytolytic activities, possibly due to changes in NK cell biology or activation (Vermeulen et al., 2013). It is clear that the picture of FIP pathogenesis is still complex.

What is known is that the infected monocytes/macrophages activation start a sequence of events that, depending on the host immune response, will determine the manifestation of the disease.

Pathological findings

A strong humoral immune response causes the effusive (or 'wet') form of FIP. The pathological features of effusive FIP were firstly linked to a type III hypersensitivity. The immune-complexes resulting from the linking of antibodies with viral particles and viral laden macrophages cause an Arthus-like reaction, associated with the deposition of the complexes around small venules, which trigger the release of macrophage produced factors that cause tissue damages and the development of an inflammatory process characterized by pyogranulomas, vasculitis, necrosis and effusions (Weiss and Scott, 1981; Pedersen, 2009). The activation of macrophages, mediated by the production of IFN- γ , results in the increased viral replication which, in turn, further fosters the inflammatory process (Berg et al., 2005). It was also recently demonstrated that FIPV-infected macrophages induce the endothelial cells to express P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) which, in turn, support the adhesion of leukocytes to endothelial cells and their transmigration. This could explain not only the systemic spread of FIP lesions, but could also give further insights about the development of typical FIP lesions (Acar et al., 2016). Some aspects of FIP lesions do not correlate with type III hypersensitivity, like the involvement of arteries in the inflammatory process. FIP vasculitis, in fact, is usually confined to small and medium size veins (Kipar et al., 2005).

Pyogranulomas are considered the histological hallmark of effusive FIP. Their localization follows the course of the cranial mesenteric artery and the lesions are therefore more concentrated on the omentum and on the abdominal serosal surfaces, but pleura and pericardium can also be involved. Histologically, pyogranulomas are composed by a high number of macrophages containing a large amount of internalized viruses, few surrounding plasma cells, lymphocytes and a variable number of neutrophils depending on the amount of necrosis (Kipar et al., 1998; Berg et

al., 2005; Pedersen, 2009). Effusive FIP is also associated with the presence of large volumes of a yellow, thick, proteinaceous effusion rich in plasma proteins and hemoglobin breakdown products, often containing fibrin clumps (Drechsler et al., 2011). Effusion is typically abdominal, but the thorax and, less frequently the pericardium, can also be involved (Hartmann, 2005). A critical role in the effusive form is played by the vascular endothelial growth factor (VEGF) produced by FIPV infected macrophages, which seems to induce hyperpermeability of feline vascular endothelial cells (Takano et al., 2011).

The different pathogenesis of non effusive FIP reflects also the different pathological features, characterized by granulomas. Type IV sensitivity may play a role in the development of non effusive, granulomatous FIP lesions, due to the presence, in the focal lesions, of CD4+ lymphocytes, neutrophils and macrophages (Paltrinieri et al., 1998). Compared with pyogranulomas, macrophages in granulomas contain less or none viral antigen (Kipar et al., 1998; Pedersen, 2009). Abdominal and thoracic lesions of non effusive FIP probably originates from the serosa/pleura and then infiltrate the underlying parenchyma (Pedersen, 2009). Eyes and central nervous system (CNS), with lesions on the uvea and meninges/ependyma respectively, are usually more interested than in the effusive form of FIP (Drechsler et al., 2011).

The effusive and non effusive form are not always two pathological distinct forms, since at gross examination the effusions can be present along with granulomatous lesions typical of non effusive FIP. Also, vasculitis, which should be more typical of effusive FIP, can be observed in kidneys with granulomatous lesions caused by non effusive FIP (Berg et al, 2005; Kipar and Meli, 2014).

Uncommon manifestations of FIP are also described. Intestinal solitary lesions due to FIP, manifesting as unusually enlarged lymph-nodes or as intramural, solitary masses in the colon or in the ileocecolic junction, resembling a neoplasm, have been described (Harvey et al., 1996; Kipar et al., 1999). An uncommon “encapsulation” of the pleural effusion, resembling a mediastinal cyst, was also described (Vigani, et al., 2009). Peritonitis can involve the tunica vaginalis, causing scrotal enlargement, and testicles or penis can be involved in the inflammatory process as well (Pedersen, 1995; Sigurðardóttir et al., 2001; Rota et al., 2008). Skin lesions caused by FIP have been also described (Trotman et al., 2007; Declercq et al., 2008; Bauer et al., 2013).

Clinical and diagnostic features

The diagnosis of FIP is a very important step, both for the unavoidability of the poor prognosis and for the psychological and economic impact on owners and cat breeders. Nevertheless, this step is still considered challenging and even frustrating for the veterinarian, since at this day there is still not a gold standard for the diagnosis *in vivo* (Pedersen, 2014b). One of the main reason for this, is the impossibility to distinguish between the FECV and the FIPV either using serology or using polymerase chain reaction (PCR)/sequencing-based approaches and the fact that the majority of cats (i.e. also those with symptoms consistent with FIP but due to diseases other than FIP) is infected with the FCoV makes the picture even more complex (Addie et al., 2009; Pedersen, 2014b). Another important reason is the non specificity of the clinical signs, especially in the non effusive form, in which the symptoms are correlated with the organs involved by the granulomas. For these reasons, the diagnosis of FIP must be based on a detailed signalment and anamnesis and on a panel of laboratory tests that sometimes can be not conclusive.

FIP is more frequent in young cats, usually between 4 and 36 months and typically under 2 years of age (Norris et al., 2005; Pedersen, 2014b; Riemer et al., 2015). Typical and non specific signs are persistent, antibiotic unresponsive fever, lethargy, general discomfort, jaundice, inappetence and weight loss. Sometimes, however, signs can be absent and FIP lesions can become apparent only during routine surgery (Addie et al., 2009; Pedersen, 2009). In the effusive form, ascites with abdominal distension is the most common finding. Pleural effusion, alone or in concomitance with ascites, can cause dyspnea (Addie et al., 2009). Signs of non effusive FIP depend on which organ is affected by the lesions. Abdominal palpation may reveal just a thickened intestinal area or lymph nodes enlargement, while sometimes it can reveal the presence of granulomatous lesions as irregularities on the kidneys surface (Drechsler et al., 2011). Ocular involvement is frequent in non effusive FIP, and it manifests with retinal vasculitis, with perivascular cuffing of inflammatory cells and sometimes retinal detachment, and uveitis, with fibrin and keratic precipitates in the anterior chamber (Maggs, 2009; Stiles, 2014). Neurological signs are also more frequent in non effusive FIP, reflecting the type and scale of involvement of the brain, meninges and spinal cord. Consequently, the range of clinical signs is wide and comprises behavioural changes, hyperesthesia, cranial nerves abnormalities, caudal paresis, ataxia and seizures (Foley et al., 1998; Gunn-Moore and Reed, 2011). After the emission of a clinical suspicion of FIP, the clinician must deal with a range of laboratory tests, more vast in effusive FIP, that might increase the odds of making the right diagnosis.

Hematology and serum biochemistry

Non regenerative anemia, ascribable to a chronic inflammatory illness, is usually found in FIP.

Other complete blood count (CBC) alterations are leukocytosis with absolute neutrophilia and lymphopenia. These two findings, however, can also be found in the stress leukogram that can be due to many other diseases (Paltrinieri et al., 2001; Addie et al., 2009; Pedersen, 2009). In a recent study performed on a large cohort of FIP cats, lymphopenia was observed in only half of the cats and significantly more often in cats with effusive FIP (Riemer et al., 2015).

In serum, an increase in hepatic enzymes, urea and creatinine may reflect the involvement of liver and kidneys, if present, but these changes are usually not helpful in formulating a diagnosis (Addie et al., 2009). Hyperbilirubinemia and sometimes hyperbiliverdinemia are often present, not in correlation with jaundice or liver involvement. These findings are more common in the effusive form and reflect the recycling of hemoglobin products derived by the leakage and destruction of erythrocytes in the effusions and lesions (Pedersen, 2009).

More useful information for the diagnosis of FIP can be obtained from the analysis of serum proteins. One common finding is hyperproteinemia, generally caused by the increase of the γ -globulins concentration. This finding, however, is common also in diseases other than FIP, and was found in low percentages of FIP cats when a large number of cases was analyzed (Paltrinieri et al., 2002; Norris et al., 2005; Tsai et al., 2011; Riemer et al., 2015). One more consistent finding is the decrease of the albumin:globulin (A:G) ratio, which was described to have a greater diagnostic value in comparison with total proteins and γ -globulins concentration increase and, when lower than 0.8, a good predictor for FIP (Hartmann et al., 2003). The A:G ratio, however, must be considered in relation with the disease prevalence, since in a population with a low prevalence of FIP, the negative predictive value (NPP) is high, allowing to exclude the disease, while the positive predictive value is very low even at a cut-off of 0.6 (Jeffery et al., 2012). Other consistent findings are changes in the globulin concentrations and typical electrophoretic patterns since during FIP, both α_2 and γ -globulins concentrations increase and the electrophoretogram usually reveals polyclonal hypergammaglobulinaemia as well as an increase in α_2 fraction (Stoddart et al., 1988; Sparkes et al., 1991; Paltrinieri et al., 1998; Paltrinieri et al., 2001; Addie et al., 2009; Taylor et al., 2010; Riemer et al., 2015). Hypergammaglobulinemia is not related to an increase of the antibodies levels but probably with the increase of some serum proteins with γ motility, probably complement factors, while the increase of the α_2 fraction is due to the presence of high levels of acute phase proteins, mostly haptoglobin (Stoddart et al., 1988; Paltrinieri et al., 1998; Pedersen,

2009). A Recent study found an increase of the γ -globulin concentrations, manifested as polyclonal gammopathy, in most of FIP cats, while only a small percentage of cats showed an increase of the α_2 -globulins concentrations (Taylor et al., 2010).

The acute phase protein α_1 -acid glycoprotein (AGP) is a very useful marker for the diagnosis of FIP. Extensive studies have been performed on its concentration in FIP cats and in cats from multi-cat environments exposed to feline coronavirus after its increase was found in experimentally induced FIP (Stoddart et al., 1988; Duthie et al., 1997). When FIP appears in a multi-cat environment, there are fluctuations of acute phase proteins and especially of AGP in healthy cats, maybe as a sign of inflammatory response in reaction to the presence of FCoV mutants in the environment, while in cats that develop the disease, the AGP concentrations remain high (Giordano et al., 2004). Being a protein that can increase due to diseases other than FIP, the diagnostic value of AGP for FIP was assessed by several studies. According to Duthie et al. (1997), an AGP concentration of 1.5 mg/mL supports the diagnosis of FIP. A further study demonstrated that an AGP concentration of 1.5-2 mg/mL can be helpful in discerning between FIP and cats with other diseases when the anamnesis and clinical features are consistent with FIP, while a concentration above 3 mg/mL can support the diagnosis of FIP even if the pre-test probabilities are low (Paltrinieri et al., 2007). In a recent study, a small number of clinical challenging FIP cases was analyzed, and AGP was in perfect concordance with histopathology (Giori et al., 2011). Besides increased its concentrations, AGP is also hyposialylated in FIP cats compared with healthy cats or cats affected with other diseases, down-regulating in this way the phagocytic activity of feline neutrophils and probably contributing to the cell-mediated immune response deficit typical of FIP (Rossi et al., 2013).

Analysis of effusions

Effusions should always be evaluated, since tests on effusions have a higher diagnostic accuracy compared with tests on blood (Hartmann, 2005; Addie et al., 2009). FIP effusions are usually cloudy, yellowish and with egg-white consistency, often presenting fibrin clots or strands (Pedersen, 2014b). Protein content is high (>3.5 g/dL), while cellular content is variable, but usually low (<5000 white blood cells/ μ L). At cytology, a mixed population of not nerated neutrophils, lymphocytes, macrophages and mesothelials cells on a granular proteinaceous background is usually seen. Cytology has a good sensitivity but a moderate specificity, since the FIP pattern is common of non specific inflammatory processes (Paltrinieri et al., 1999). On the other

hand, in cats with diseases other than FIP it allows to quickly recognize septic or neoplastic effusions, therefore it should be always performed (Addie et al., 2004). The Rivalta's test, a test developed a century ago and initially used to differentiate exudates from transudates in human body cavity effusions, may be useful to identify FIP effusions. In the presence of high protein concentrations and probably of inflammatory mediators typical of FIP, in fact, the effusions form a visible clot when added to an acetic solution. The Rivalta's test diagnostic accuracy was recently re-discussed, and found lower than previously reported (Hartmann, 2005; Fischer et al., 2012). Moreover, the test is subjective and operator-dependent (Fischer et al., 2013).

Serology

The main issue with antibodies detection, is that FECV and FIPV are antigenically identical therefore causing the same antibody response (Pedersen, 2009). Therefore, FCoV-infected, healthy cats, can result positive at serological tests (Addie et al., 2009). Titers above 1:1.600, in combination with other findings suggestive of FIP, may increase the odds of diagnosing FIP. Cats from environments where the FCoV is endemic, however, may have high titers as well. Values above 1:3.200 are considered highly suggestive of FIP, but also in these cases serology should always be evaluated in combination with other tests (Hartmann et al., 2003; Hartmann et al., 2005; Pedersen, 2014b). Another issue is the low sensitivity of serological tests. This finding was recently ascribed to the presence of high viral load that would bind the antibodies, making them no more available for the test. However, sera and effusions with low coronavirus antibody titers were positive at PCR, making this explanation not sufficient for the low sensitivity of serology (Meli et al., 2012).

Viral detection

Immunohistochemical demonstration of the antigen inside the lesions is considered the gold standard for the diagnosis of FIP (Tammer et al., 1995). There are, anyway, some cases where it can be falsely negative, depending on the quality of tissues, reagents and quantity of infected macrophages (Pedersen, 2014b). Immunocytochemistry can be performed also *in vivo* on biopsies or fine needle aspirates (FNAs), but the sensitivity is influenced by the type of sampling, the type of lesion and the cellularity. The sensitivity, however, can increase when biopsies and FNAs from the same tissue are considered together (Giordano et al., 2005). Immunocytochemistry or immunofluorescence on effusions have been always considered helpful to confirm the disease but

not to exclude it, since this test is influenced by the cellularity of the sample and by the amount of virus in the macrophages (Paltrinieri et al., 1999; Hartmann et al., 2003; Addie et al., 2004). Recent studies, however, have shown for these tests a specificity lower than previously reported, possibly due to the leakage, in presence of inflammation, of FCoV infected monocytes in the effusions (Litster et al., 2013; Felten et al., 2016).

Polymerase chain reaction (PCR) is a direct test with undeniable usefulness for the detection of shedders when the test is performed on the feces (Addie et al., 2004). Nonetheless, its value for the diagnosis of FIP is still argument of debate. In fact, while the PCR on feces is known to have no diagnostic value for FIP, its application on body fluids has been recently discussed. PCR on cerebrospinal fluid (CSF) and on other body effusions has shown optimal specificity but a low or suboptimal sensitivity, showing this to be a good test to confirm FIP but not to exclude it (Pedersen et al., 2014b; Doenges et al., 2015; Longstaff et al., 2015; Doenges et al., 2016). It is also known that FIP cats may have low or absent viral load in blood, and only 44% of suspected FIP cases have been recently shown to be PCR positive on effusions (Herrewegh et al., 1995b; Addie et al., 2004; Soma et al., 2013).

The spike protein gene sequencing proposed by Chang et al. (2012) has been recently described by Kipar and Meli (2014) as “one of the most diagnostic potential tool involving the direct virus detection” and it is currently used by some laboratories for the diagnosis of FIP, even though only two studies about its diagnostic accuracy have been published. In particular, the diagnostic accuracy of the sequencing aimed to detect the M1058L mutation was investigated on effusion samples. While in one study the specificity could have not been investigated, another study showed an absolute specificity. On the other hand, the sensitivity resulted similarly low (Felten et al., 2015; Longstaff et al., 2015).

FIP management

Therapies

A large number of different therapies have been attempted over the years for the treatment of FIP, but to this day none has been proven effective. Three main approaches have been used: inhibition of viral replication, inhibition of the inflammatory response and non specific modulation of the immune system (Pedersen, 2014b). Viral encoded proteases that cleave viral polyproteins into functional single proteins are the target of recently discovered anti viral drugs. In a very recent study, a 3C-like (3CLpro) protease inhibitor that previously showed anti-FIPV *in vitro* was

tested *in vivo* in experimentally induce FIP. The treatment caused the regression of clinical symptoms and the reduction of viral load in macrophages from ascites in 6/8 cats (Kim et al., 2013; Kim et al., 2015; Kim et al., 2016). Three small molecule inhibitors (chloroquine, mefloquine, and hexamethylene amiloride) demonstrated a marked inhibitory effect on FCoV replication *in vitro* but not yet *in vivo*, except for chloroquine, which demonstrated some improvement in the clinical signs of FIPV infected cats, but caused hepatotoxicity at the same time (Takano et al., 2013; McDonagh et al., 2014). Human IFN- α and - β inhibit FIPV *in vitro*, and feline IFN- ω has the same effect without causing the production of antibodies against the molecule, as it happens with the human interferon (Hartmann et al., 2005). Feline IFN- ω was at first reported to induce remissions in the majority of FIP cats, but in a study involving a larger group of cats, IFN- ω showed no advantage effect in treating FIP (Ishida et al., 2004; Ritz et al., 2007).

Prednisolone was and still is one of the classic treatment of choice when there is the suspicion of FIP, but it does not treat the disease (Addie et al., 2009). TNF inhibitors have been used to limit the inflammatory and pro-vasculitis effects of this cytokine, but with no difference neither in the survival time or in the amount of the effusions between treated and untreated cats (Fischer et al., 2011). In a more recent study, the administration of anti-feline TNF- α monoclonal murine antibodies increased the survival time of 2/3 FIP experimentally induced cats. The cats, however, may develop anti-mouse antibodies causing an anaphylactic reaction, therefore the authors started to develop a mouse-feline chimeric antibody against TNF- α (Doki et al., 2016).

A wide number of different immune stimulant compounds (staphylococcal A protein, *Propriobacterium acnes*, mucopolysaccharide extract of *Aloe vera*, a lymphocyte T cell immunomodulator and Polyprenyl immunostimulant) have been used, often off label and without evidence of a real benefit for the cat (Pedersen, 2014b).

Prevention

Due to the numerous failed attempts of developing an effective vaccine, the key for preventing FIP is to avoid FCoV infection through identification and isolation of the shedders and through the hygiene of the environment since the higher the proportion of shedding coronavirus at a given time, the more likely cats will develop FIP (Foley et al, 1997). Multi-cat environments (e.g. breeding catteries, shelters, multi-cat households) are undoubtedly high-risk environments, where infected cats can shed the virus for months or even life-long and where stress deriving from overcrowding or concurrent diseases is bigger (Addie et al., 2003; Pedersen, 2009). One way to

prevent FCoV infection is the strict quarantine of queens 2 weeks before birth and the separation and early weaning of kittens around 5-6 weeks of age, before the decrease of maternal antibodies. If kittens do not have antibodies at 12 weeks of age, they can be considered free from infection (Addie et al., 2004; Pedersen, 2009). This procedure, however, is not simple due to the strict control of exposure, to the costs for assuring enough free spaces for quarantine and for the potential problems of socialization associated with early weaning. Moreover, FCoV free kittens, could be easily become infected as soon as they are placed in another environment contaminated by FCoVs (Addie et al., 2004, Hartmann, 2005).

Another method to control the infection is to separate the shedders from negative cats. Shedders can be detected with PCR on feces, but multiple testing every 2 weeks for two months or more is necessary due to the nature of FCoV shedding (Addie et al., 2001). An indirect way to identify the shedders is the antibody testing, since cats with low antibody titers (e.g., 1:25 or below) usually, but not always, are shedding very low or no virus, while cats with high antibody titers (e.g., 1:400) do. As with PCR, repeat testing is necessary to keep track of the intermittent shedders (Hartmann et al., 2005; Pedersen, 2014b).

AIMS

Aims of the thesis

The general aim of this thesis was to contribute to the development of new tests for the diagnosis *intra-vitam* of feline infectious peritonitis and feline coronavirus infections (including the collection of updated information regarding the diagnostic potential of pre-existing clinicopathological tests) and, to a lesser extent, to deepen some aspects of feline infectious peritonitis pathogenesis.

Specific aims

- I. To determine the diagnostic accuracy of the Δ total nucleated cells (Δ TNC) measurement, which is the ratio between the two total nucleated cell counts (TNCC) in the DIFF and BASO channels of the hematology laser counter Sysmex XT-2000iV, for FIP effusions. The Δ TNC is based on the same principle of the Rivalta test, which is subjective and biased by several pre-analytical or analytical factors. The availability of a more objective approach such as the Δ TNC would improve the ability to correctly classify effusions as consistent with FIP.
- II. To determine if the Δ TNC measurement can be performed on supernatants of FIP effusions after the addition of feline blood and to assess the diagnostic accuracy of this method, as well as the influence of protein content, total cell count of the added blood and magnitude of dilution. The development of such a modified technique would in fact allow to investigate the Δ TNC also on frozen supernatants, thus allowing the collection of accurate diagnostic information also when samples cannot be immediately analyzed.
- III. To investigate the atypical serum electrophoretic (SPE) patterns recorded in FIP cats in the recent years and to compare them with SPE patterns from FIP and non FIP cats in a less recent time span. This information would be particularly useful since SPE is considered one of the most reliable tests to support a clinical diagnosis of FIP. However, anecdotal reports suggest that this diagnostic potential is diminished in recent years, due to the frequent occurrence of “false negative” results (i.e. electrophoretograms not consistent with FIP in cats actually affected by this disease).
- IV. To compare the diagnostic performances of clinico-pathological and molecular tests (in particular: SPE, α 1-acid glycoprotein - AGP - measurement, RT-nPCR and spike gene sequencing on blood; cytology, evaluation of the Δ TNC, RT-nPCR 3'UTR and spike gene sequencing on cavitory effusions and RT-nPCR and spike gene sequencing on tissues specimens) for the *ante-mortem* diagnosis of FIP. The rationale of this comparison is that despite the numerous studies published in recent

years on each of these techniques, no studies have compared the diagnostic potential of the different tests. This information would be extremely useful to know the clinical scenarios on which each of the test may provide the most reliable diagnostic information.

- V. To develop a reverse transcriptase loop mediated isothermal amplification (RT-LAMP) for the detection of FCoV in the specimens most frequently used in clinical practice for both screening and diagnostic purposes. This method is more rapid and cheap than conventional PCR and therefore it would facilitate the extensive use of molecular techniques to investigate the presence of coronavirus genomes in different clinical specimens.

- VI. To sequence a wide number of FCoVs obtained from different specimens (tissues, body fluids, feces) of histochemically confirmed FIP and non-FIP cats and to better understand the correlation between the spike gene mutations described by Chang et al. (2012) and FIP development. Sequences of viral strains collected from cats with and without FIP are widely investigated in recent times but often performed on a limited number of samples. The rationale of this specific aim is that any additional information on the genomic variability of viral strains may be relevant in terms of basic knowledge of host-virus interaction.
 - VI.1. To assess possible differences in the frequency of FCoV shedding in cats affected or non affected by FIP, and to investigate any spike protein mutations present. Also in this case, this information would enrich the currently available information on the characteristics of viral shedding and it may be a preliminary step for future studies on the development of diagnostic tests or of tests potentially applicable in pathogenic studies.

DESCRIPTION OF STUDIES I-VI

I. High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis

Material and methods

Case selection

This retrospective study was performed on data obtained from effusions submitted for diagnostic purposes and under informed consent of the owners to the Department of Veterinary Medicine of the University of Milan (DIMEVET). Feline intracavitary effusions analyzed with the Sysmex XT-2000iV from June 2009 to June 2013 were selected from the DIMEVET database and used for data analysis if fulfilling the criteria reported in the following table 1.

Inclusion criteria	Exclusion criteria
Available complete documentation of physico-chemical analysis of the effusion	Absence of follow-up
Available extensive information about the final diagnosis	Absence of cytologic information
Available cyto-centrifuged slides in the archive of DIMEVET for those cases where no cytologic report was available	Absence of slides to verify the cytologic pattern in cases without cytology report in the database.
	Unclear or non-conclusive cytologic findings in cases with archived slides but without information in the database

Table 1. Inclusion and exclusion criteria used for this study.

Cats were defined as FIP if histopathology was consistent with FIP and if immunohistochemistry against FCoV was positive. Cats were considered as non-FIP if another disease was diagnosed through effusion analysis, eventually confirmed by necropsy and histology, or for clinical improvement after treatment.

Sysmex XT-2000iV

Only effusions collected in EDTA tubes and submitted no later than 12–18 hours after sampling were analyzed on the Sysmex XT-2000iV (Sysmex Europe GmbH, Norderstedt, Germany) analyzer to determine TNCC provided by both the DIFF (TNCC-DIFF) and BASO (TNCC-BASO) channels, as well as the Δ TNC. Specifically, the DIFF channel classifies all nucleated cells based on complexity

and nucleic acid content. The BASO channel classifies nucleated cells based on volume and the complexity of cellular residues produced after contact with an acidic reagent that, in human blood, condenses all the nucleated cells except basophils (Lilliehook and Tvedten, 2011). Since effusions include cells other than WBC, the total WBC count and the Δ WBC generated by the instrument were defined as TNCC and Δ TNC.

Statistical analyses

The Analyse-it software (Analyse-it Software Ltd, Leeds, UK) was used. A nonparametric t-test (Mann–Whitney U-test), using the 95% confidence interval (CI) as a measure of uncertainty was used to compare to each other TNCC-DIFF, the TNCC-BASO, and Δ TNC results from FIP and non-FIP cats. To assess the diagnostic accuracy of Δ TNC, the number of true positive (TP), false-positive (FP), true-negative (TN), and false-negative (FN) results were calculated as follows: TP, effusions from FIP cats with a Δ TNC higher than each operating point; TN, effusions from non-FIP cats with a Δ TNC lower than each operating point; FP, effusions from non-FIP cats with a Δ TNC higher than each operating point; and FN, effusions from FIP-cats with a Δ TNC lower than each operating point. Sensitivity and specificity were calculated using standard formulas and using the 95% CI as a measure of uncertainty (Christenson, 2007). Positive and negative likelihood ratios (LR+ and LR-, respectively) were calculated using the formulas: $LR+ = \text{sensitivity}/(1-\text{specificity})$ and $LR- = (1-\text{sensitivity})/\text{specificity}$, respectively (Gardner and Greiner, 2006).

Finally, receiver operating characteristic (ROC) curves were designed by plotting sensitivity vs 1-specificity to determine the discriminating power of Δ TNC in identifying cats with FIP (Gardner and Greiner, 2006). The optimal cut-off value was identified as the operating point closer to the upper left corner of the graph.

Analytical precision and accuracy

Since there is no information about repeatability and linearity under dilution of Sysmex readings of TNCC-DIFF and TNCC-BASO of effusions from cats with FIP, in the current study, repeatability was assessed on 2 FIP samples with a high Δ TNC and on 2 specimens with a normal Δ TNC in 5 consecutive measurements on one day, and by calculating the CV with the formula: $CV = \text{mean}/SD \times 100$. To assess linearity under dilution, one specimen with high and one with normal Δ TNC were each serially diluted at 1:1, 1:3, 1:7, and 1:15 (vol/vol) with isotonic saline, resulting in dilutions corresponding to 50%, 25%, 12.5%, and 6.25% of the original effusion, respectively. After being

analysed on the Sysmex, linearity was determined by comparing the expected values for each dilution to the values provided by the instrument in a linear regression analysis.

Results

Retrospective study population and distribution of cases per group

The results of the retrospective search are reported in figure 1. The 51 selected effusions were grouped as follows:

Group A, FIP (20 cats). In all these cases except 2, the physico-chemical features and cytology of the effusions were consistent with FIP. The 2 cases of FIP with “atypical” findings in the effusion included cat 5 with polyclonal gammopathy and very high serum AGP concentration, but an effusion with a low protein concentration (17 g/L), low specific gravity (1.010), and low cellularity ($0.13 \times 10^9/L$), with rare neutrophils and mesothelial cells in the absence of the typical proteinaceous background. The pericardial effusion of cat 25 revealed a high number of reactive mesothelial cells, sometimes with evident cytophagia and a weakly proteinaceous background. However, in all the 20 cases, the diagnosis of FIP was confirmed by histology and by the immunohistochemical detection of intralesional FCoV.

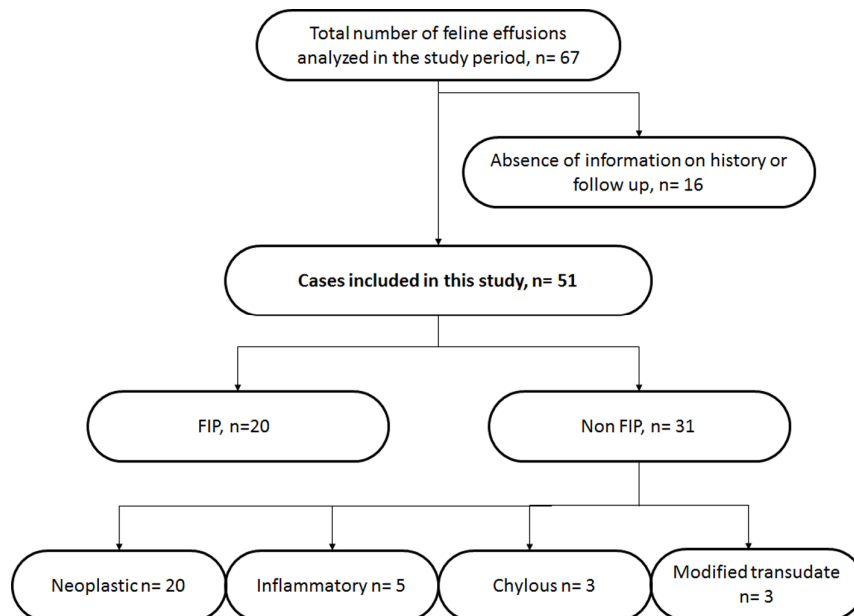


Figure 1. Inclusion and exclusion criteria adopted for the selection of cases from the database and the final assignment to the study groups.

Group B, non-FIP (31 cats). Details about the diagnosis of each cat are reported in table 2.

Unfortunately, the non-FIP group contained a high number of neoplastic effusions that were not as challenging for a differential diagnosis from FIP, being easily recognizable by cytology.

Type	n°	Subtype	Diagnosis
Tumours (20)	10	Lymphomas	Cytology and imaging
	8	Epithelial tumors	Cytology and imaging
	1	Thymoma	Cytology and imaging
	1	Hemangiosarcoma	Cytology, imaging, PM, histology
Inflammatory (5)	3	Septic	Bacteriology
	2	Cholangiohepatitis	Laboratory findings
Chylous	3	Heart failure	Cytology and imaging
Modified transudate (3)	2	Abdominal neoplasia	PM, histology
	1	Cardiomyopathy	Imaging, remission after treatment

Table 2. Subdivision of the non FIP effusions in the different subtypes and diagnostic methods adopted. PM: post mortem examination.

Repeatability and linearity under dilution

Repeatability of specimens with normal Δ TNC was better for both DIFF and BASO counts as well as for Δ TNC, with CV < 2.56%. Conversely, CV was higher and extremely variable for the specimens with high Δ TNC, due to a high variability in both BASO and DIFF counts (table 3). Linearity under dilution provided excellent results for TNCC-DIFF and TNCC-BASO of the specimens with normal Δ TNC, with correlation coefficients of 0.99 and 1.00, respectively ($P < 0.001$). Consequently, Δ TNC remained constant over the different dilutions. Conversely, the linearity under dilution of specimens with high Δ TNC was satisfactory only for DIFF-TNCC (table 4, figure 2,3).

	Repeatability											
	Cat #1 (FIP)			Cat # 2 (FIP)			Cat # 2 (lymphoma)			Cat # 4 (Inflammation)		
	DIFF	BASO	Δ TNC	DIFF	BASO	Δ TNC	DIFF	BASO	Δ TNC	DIFF	BASO	Δ TNC
Run 1	1.47	0.11	13.364	0.72	0.09	8.000	5.59	5.58	1.002	12.41	11.98	1.036
Run 2	2.01	0.13	15.462	0.81	0.05	16.200	5.48	5.66	0.968	12.19	12.04	1.012
Run 3	2.05	0.12	17.083	0.74	0.05	14.800	5.58	5.55	1.005	12.62	12.04	1.048
Run 4	2.14	0.14	15.286	0.79	0.07	11.286	5.57	5.63	0.989	12.59	11.96	1.053
Run 5	2.45	0.12	20.417	0.78	0.07	11.143	5.36	5.67	0.945	12.54	12.21	1.027
Mean	2.02	0.12	16.32	0.77	0.11	12.29	5.52	5.62	0.98	12.47	12.05	1.04
SD	0.35	0.01	2.64	0.04	0.02	3.25	0.10	0.05	0.03	0.18	0.10	0.02
CV (%)	17.52	9.19	16.18	4.82	15.21	26.47	1.77	0.92	2.56	1.41	0.82	1.57

Table 3. Results regarding repeatability recorded in two cats with high Δ TNC associated with FIP and in two cats with normal Δ TNC.

	Linearity under dilution					
	Cat # 2 (FIP)			Cat # 2 (lymphoma)		
	DIFF	BASO	Δ TNC	DIFF	BASO	Δ TNC
Undiluted	0.97	0.11	8.818	5.67	5.53	1.025
50%	0.48	0.1	4.800	2.54	2.54	1.000
25%	0.2	0.11	1.818	1.05	1.16	0.905
12.5%	0.21	0.1	2.100	0.67	0.73	0.918
6.25%	0.12	0.12	1.000	0.34	0.4	0.850

Table 4. Results regarding linearity under dilution recorded in a cat with high Δ TNC associated with FIP and in a cat with normal Δ TNC.

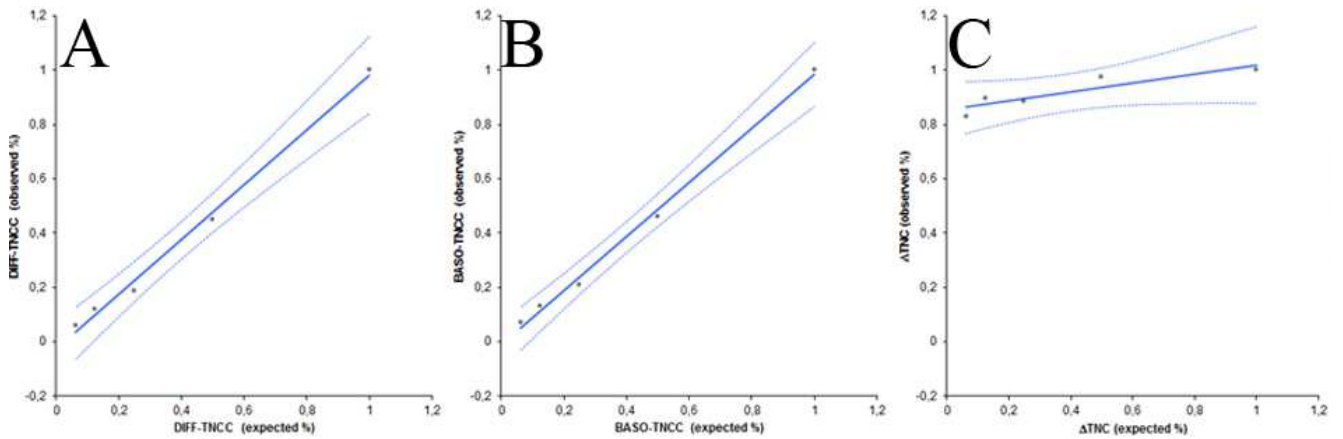


Figure 2. Linearity under dilution (LUD) recorded in serially diluted effusion specimens from a cat with lymphoma (A, DIFF-TNCC; B, BASO –TNCC; C, Δ TNC). Values on the y-axes refers to observed values, while values on the x-axes refers to expected values.

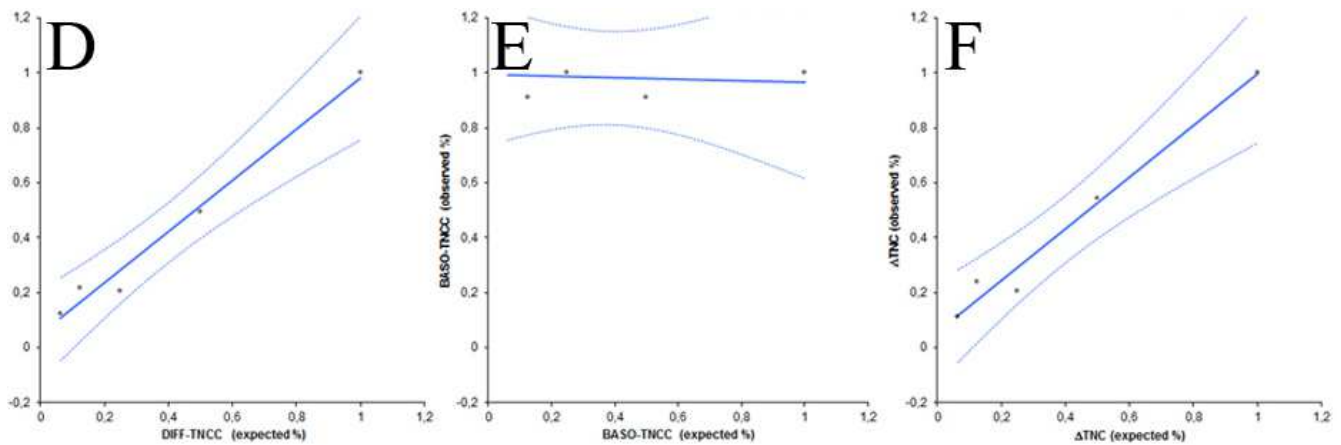


Figure 3. Linearity under dilution (LUD) recorded in serially diluted effusion specimens from a cat with FIP (A, DIFF-TNCC; B, BASO –TNCC; C, Δ TNC). Values on the y-axes refers to observed values, while values on the x-axes refers to expected values.

Comparison of TNCC-DIFF, TNCC-BASO, and Δ TNC between cats with and without FIP

The TNCC-BASO and TNCC-DIFF counts were significantly lower ($P < 0.001$ and $P < 0.05$, respectively) in cats with FIP (TNCC-BASO = 0.2; 0.0–5.3; TNCC-DIFF = 1.5; 0.1–26.3) than in non-FIP cats (TNCC-BASO = 10.1; 0.0–707.9; TNCC-DIFF = 9.1; 0.1–921.8; Figure 4), with a high inter-individual variability in the latter group, likely due to the heterogeneity of the diseases. The Δ TNC was significantly higher ($P < 0.001$) in cats with FIP (median: 9.3; min–max: 0.5–36.4) than in non-FIP cats (1.0; 0.5–2.5). All the cats with FIP had a Δ TNC > 3.0 , except for the 2 “atypical” FIP effusions (cat 5, Δ TNC 0.538; cat 25, Δ TNC 1.165). One of the cats showing “atypical” FIP effusion had hypoalbuminemia and a hemorrhagic syndrome, which are changes with liver failure that may induce also hypofibrinogenemia, likely preventing clotting in the BASO reagent. In the other case,

the cytologic pattern of the effusion was complicated by the presence of “atypical” mesothelial cells that are usually not abundant in FIP effusions. In both cases, the false-negative results of Δ TNC may be related to atypical features of the effusion rather than the low analytical sensitivity. All non-FIP cats had a Δ TNC < 3.0. More specifically, the only 2 specimens from all 31 cats without FIP with a Δ TNC > 1.7 but < 3.0 were a cat with lymphoma and a highly cellular effusion (TNCC-DIFF: 25.45 cells \times 10⁹/L), and an almost non cellular modified transudate from a cardiopathic cat (TNCC-DIFF: 0.05 cells \times 10⁹/L). Lymphomas can sometimes provide positive Rivalta’s test results in people (Sakai et al., 2004) while the high Δ TNC in the second case, was clearly a mathematical artifact due to the analytical sensitivity of the instrument.

These results demonstrate that cells are entrapped in clots formed by fibrinogen precipitation in the BASO channel, therefore cell counts in the BASO channel are usually lower than those of the DIFF channel. This mechanism explains why the Δ TNC (in the Sysmex referred to as Δ WBC) increases in such specimens.

Diagnostic accuracy of Δ TNC

The area under the ROC curve for Δ TNC (figure 5) was 0.94 (95% CI = 0.84–1.00, $P < 0.001$ compared with the line of no discrimination). The best cut-off for Δ TNC determined by the ROC curve analysis was 1.7. At this value, sensitivity was 90.0% (95% CI = 68.3–98.8%), specificity was 93.5% (95% CI = 78.6–99.2%), LR+ was 13.9 (95% CI = 4.6–86.3), and LR- was 0.11 (95% CI = 0.0–0.3). When using a cutoff of Δ TNC = 2.5, specificity increased to 100%. Thus, Δ TNC has a high diagnostic accuracy for FIP. Specifically, a Δ TNC > 1.7 makes it 14 times more likely that an effusion originates from a cat with FIP rather than a different disease. Conversely, with a Δ TNC < 1.7, the probability that the effusion comes from a cat with FIP is about one-tenth of the probability that the effusion comes from a cat with a different disease.

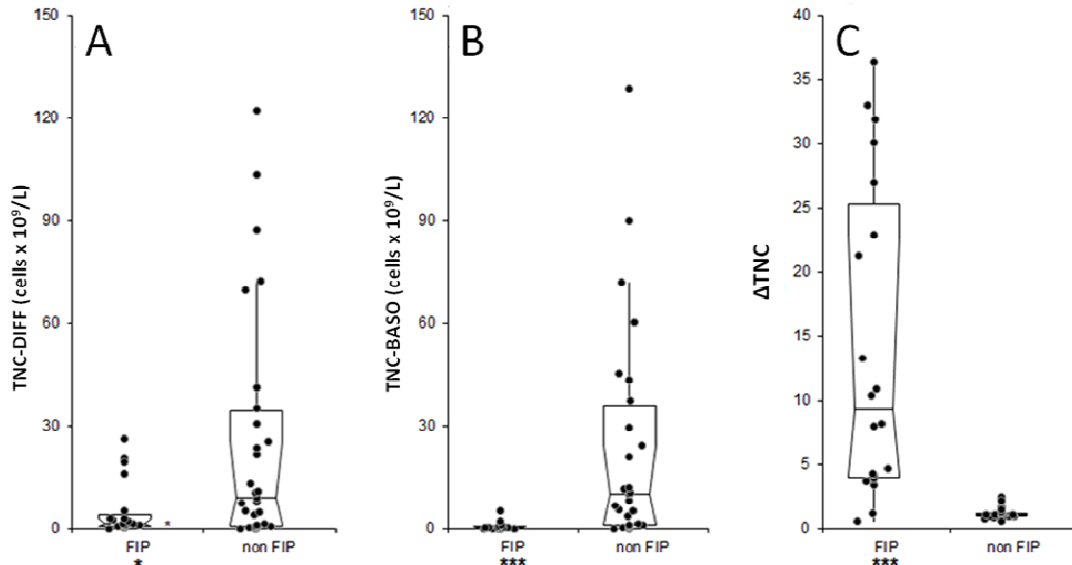


Figure 4. Values of TNCC-DIFF (A), TNCC-BASO (B), and the ratio between total nucleated cell counts in the DIFF and BASO channel of Sysmex XT-2000iV, reported as Δ WBC by the instrument but termed Δ TNC in this study (C) recorded in cats FIP and non-FIP cats. The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median, whiskers extend to within the I quartile minus 1.5*IQR or to within the III quartile plus 1.5*IQR. Dots indicate the values recorded in this study. The TNCC-DIFF and the TNCC-BASO graphs do not include the result of a neoplastic non-FIP specimen that had extremely high TNCC-DIFF and TNCC-BASO counts (921.8 and 707.9 cells x 10⁹/L). The black bolded asterisks reported below the X-axis indicate a significant difference between groups (*P < .05; ***P < .001).

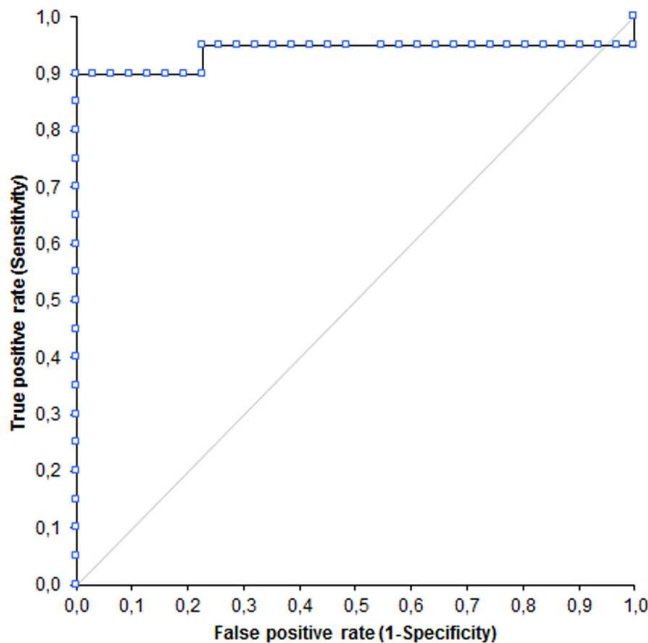


Figure 5. Receiver operating characteristic (ROC) curves of the ratio between total nucleated cells in the DIFF and BASO channel of Sysmex XT-2000iV, reported as Δ WBC by the instrument but termed Δ TNC in this study, for the diagnosis of FIP. The gray line indicates the line of no discrimination.

Discussion

The results of the current study suggest that the instrumental analysis with the Sysmex XT-2000iV may represent an additional reliable method for confirmation of FIP in the analysis of effusions. A possible limitation of this study was the low number of non neoplastic effusions that in routine practice may benefit from an additional test to distinguish FIP from other types of inflammatory or reactive effusions. However, also in a previous feline effusions study, all the specimens from cats with inflammatory effusions other than FIP had a $\Delta\text{TNC} < 1$, confirming that a $\Delta\text{TNC} > 1$ has a high diagnostic accuracy for FIP (Pinto da Cunha et al., 2009).

Independently on the few FN and FP cases, the analysis of effusions with the Sysmex XT-2000iV counter evidenced a sensitivity and a specificity comparable to or even higher than that previously reported for the Rivalta's test (Fischer et al., 2012). Moreover, the Rivalta's test is rapid, cheap, and accurate, but it may be limited due to inappropriate techniques or intrinsic factors of the reagents such as concentration of acetic acid, different temperatures of the effusion, and the acetic acid solution (Fischer et al., 2013). Additionally, the Rivalta's test provides semi-quantitative results and does not allow grading the severity of the change. Finally, the evaluation of the test is subjective and a high inter-observer variability is observed (Fischer et al., 2013). Conversely, the analysis with the Sysmex XT-2000iV counter is more standardized and rapid and provides, in addition to the cell count, a provisional information on the cell types based on the scattergram (Pinto da Cunha et al., 2009). However, it must be stressed that the linearity under dilution test performed in this study demonstrated that the more accurate cell count provided by the instrument is the DIFF-TNCC, which is not affected by the entrapment of cells in the clots formed after contact with the BASO reagent. Therefore, in routine practice, it is not recommended to use the default WBC counts that are generated by the BASO channel. Precision and linearity were not satisfactory at high ΔTNC values. However, this does not affect the clinical decision since the difference between ΔTNC values recorded in cats with and without FIP was higher than the imprecision or bias of the method.

In conclusion, this study evidenced a very high diagnostic accuracy of the Sysmex-generated ΔTNC , where values > 1.7 are highly suggestive of FIP, and a $\Delta\text{TNC} > 3.4$ which, although higher than the cut-off with the maximum specificity recorded in this study, was the threshold confirmatory for FIP in a previously published study (Pinto da Cunha et al., 2009), may be considered diagnostic for FIP.

II. Diagnosing feline infectious peritonitis using the Sysmex XT-2000iV based on frozen supernatants from cavitory effusions

Material and methods

Thirty-four feline effusions were collected for diagnostic purposes and submitted to the DIMEVET laboratory according to standard veterinary procedures, therefore without the need of a formal approval of the Institutional Ethical Committee.

Nineteen effusions were classified as FIP based on *post mortem* histology and immunohistochemistry (18/19) or through histology and immunohistochemistry of biopsies (1/19). FIP was excluded based on postmortem findings in 5 of the 15 cats without FIP. These 5 cats were diagnosed with pleuropericardial chronic fibrosis (cat 20), pleural undifferentiated pleomorphic sarcoma (cat 21), abdominal hemangiosarcoma (cat 24), undifferentiated liver neoplasia (cat 27), and acute leukemia (cat 33). In 4/15 cats without FIP, a diagnosis of neoplasia was obtained through cytology (cats 22, 23, 30, 34) and confirmed through histology (cats 30, 34). In the remaining 6 of 15 cats without FIP, the following diagnoses were obtained *in vivo*: cats 25 and 29 were diagnosed with cardiomyopathy and cat 32 with pancreatitis, all responding to the specific treatment. Cats 26, 28, and 31 were diagnosed with chylothorax through cytology and diagnostic imaging.

Twenty-seven samples were collected in EDTA tubes and analyzed with the Sysmex XT-2000iV within 12–18 h. After the Δ TNC measurement, the effusions were centrifuged ($3,000 \times g$, 5 min) and the supernatants transferred to other tubes and frozen at -20°C (~1–8 weeks). Seven samples (1, 10, 16, 18, 19, 28, 29) were centrifuged by the referring veterinarians, and the supernatants sent to our laboratory where immediately frozen (~1–8 weeks).

Twenty μL of fresh feline blood (ΔWBC of 1.00 ± 0.15 ; mean \pm standard deviation), obtained from the routine caseload of the DIMEVET laboratory, were added to 180 μL of thawed supernatants to obtain a dilution of 1:10 (figure 1).

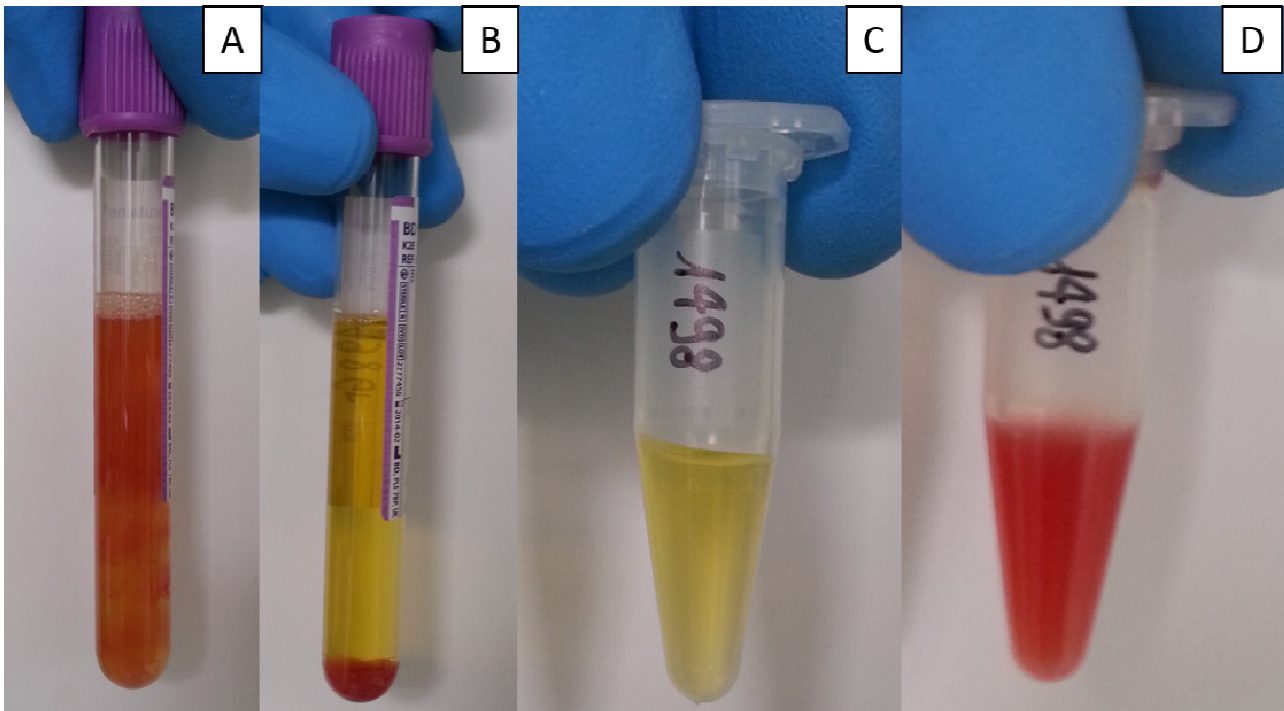


Figure 1. Procedure for the evaluation of the modified Δ TNC measurement. A. collection of the effusion in EDTA tubes. B. Centrifugation of the sample followed by the removal of the supernatant. C. Freezing and thawing of the supernatant in a other tube. D. Addition of feline blood in a 1:10 dilution.

Specifically, 15 different blood samples were used (WBC counts: $5.11\text{--}154 \times 10^9$ WBC/L). Each blood sample was used to spike 1–4 effusions (Table 1).

After gentle mixing, the samples were analyzed with the Sysmex XT-2000iV. WBC-DIFF and WBC-BASO counts and Δ TNC were recorded. Samples were then classified as positive or negative using a Δ TNC of 1.7 and a Δ TNC of 3.4 as cut-offs. Using both the cut-offs and based on the final diagnosis (FIP vs. non-FIP), the numbers of TP, TN, FP, or FN results were counted. Specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were then calculated.

Added blood sample	WBC count in added blood ($\times 10^9/L$)	FIP effusion cats	Non-FIP effusion cats
1	5.1	2, 4, 6, 7	
2	11.4	13	
3	13.0		29
4	13.4	3, 18	21, 22
5	14.1	19	26, 27, 34
6	15.3		33
7	19.4	16	20, 28
8	20.1	14	32
9	26.8	9	
10	34.1	17	
11	36.2		25
12	39.9	1, 5, 10, 15	
13	44.6	11, 12	
14	65.0	8	23, 24
15	154		30, 31

Table 1. White blood cell (WBC) counts of the 15 blood samples added to the effusions of the 34 cats included in the study.

Results and discussion

The addition of blood did not generate a matrix effect that interfered with the cell counts, as demonstrated by the fact that the WBC-DIFF in the effusion was close to 10% of the added blood cell count, as expected using the 1:10 dilution (10.9 ± 1.2 in the whole caseload; 11.1 ± 1.2 in FIP effusions; 10.8 ± 1.2 in non-FIP effusions). This was found in all the cases but one, where the WBC-DIFF count recorded in the effusions added with blood was 24.6% compared with the WBC count in blood.

In 12 of 27 cases that were analyzed with both methods (cats 2–4, 6, 7, 13, 15, 17, 21, 31, 32, 34), the modified Δ TNC was lower than the original one (Fig. 2).

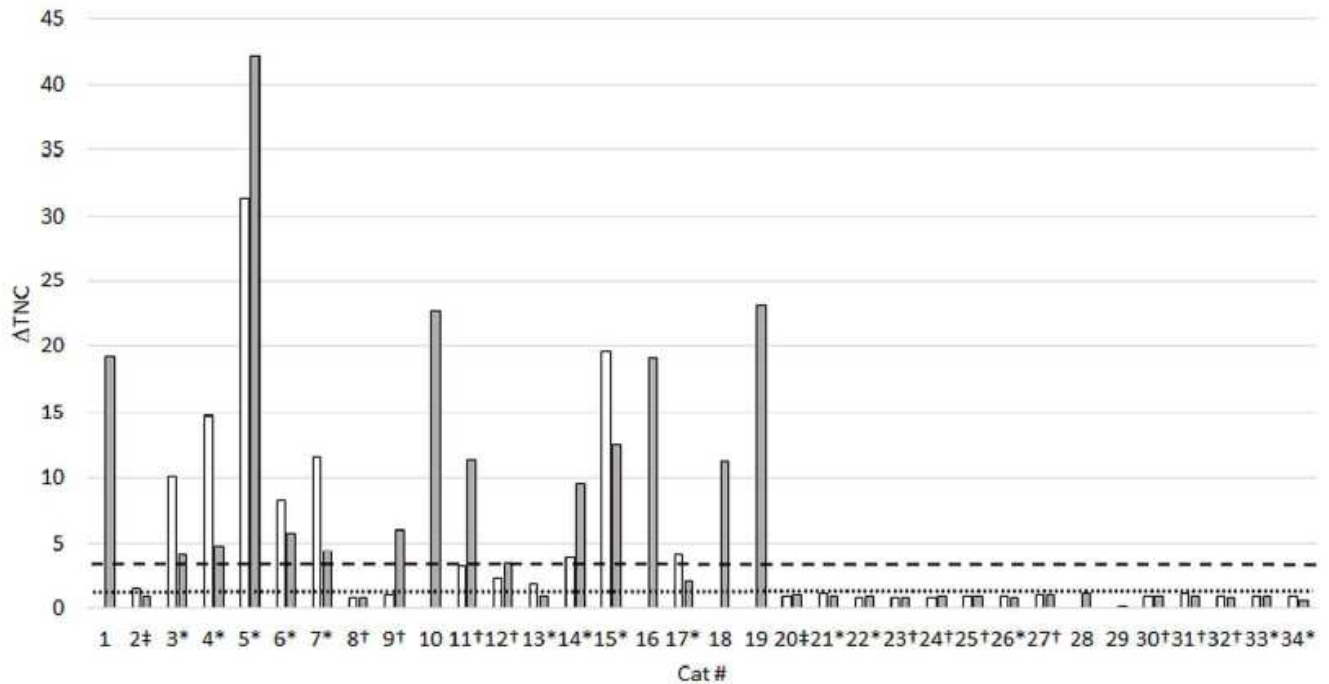


Figure 2. Results of the delta total nucleated cells (Δ TNC) on fresh effusions with the original method (white bars) and on frozen–thawed supernatants using the modified method described in this study (gray bars) in cats with feline infectious peritonitis (cats 1-19) or without FIP (cats 20-34). The horizontal dashed black line and the horizontal dotted line indicate the cutoffs that have been defined, respectively, as “suggestive for FIP” (1.7) and “consistent with FIP” (3.4) in the previous study.

* Effusions in which the WBC-DIFF count was lower in the modified method than in the original method.
 † Effusions in which the WBC-DIFF count was higher in the modified method than in the original method.
 ‡ Effusions in which the WBC-DIFF count was unchanged in the modified method compared with the original method.

In 8 of these cases, the WBC-DIFF count resulted from the addition of blood, was lower than in the fresh sample. Conversely, in 6 of 27 cases (cats 5, 9, 11, 12, 14, 26), the modified Δ TNC was higher than the original, and in 3 of these cases the WBC-DIFF count was higher than that of the fresh sample. In 9 of 27 cases (cats 8, 20, 22–25, 27, 30, 33), 8 of which belonged to the non-FIP group, the modified Δ TNC remained unchanged compared with the original Δ TNC (<10% variation compared with the original Δ TNC), despite the WBC-DIFF count being higher in 6 of these cases compared with the fresh sample. Therefore, the modified method induced variations of the Δ TNC in approximately two-thirds of samples (16 of 27 cases).

Results obtained with the 2 methods were compared using a Wilcoxon signed rank test, and no significant differences were recorded, suggesting that frozen supernatants provide results similar to fresh samples (figure 3).

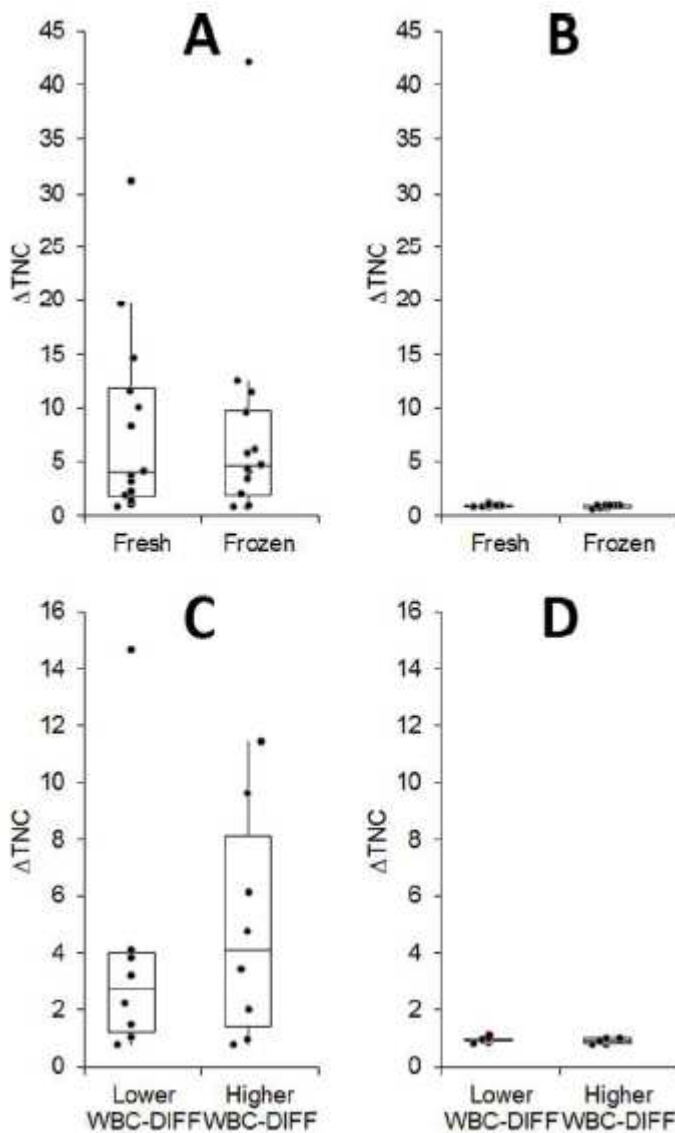


Figure 3. Comparison of results obtained in fresh effusions processed with the original method or in frozen effusions using the modified method (A and B) or in frozen samples with different blood effusion ratios (C and D). The Δ TNC recorded in cats with FIP are reported in A and C; The Δ TNC recorded in cats without FIP are reported in B and D.

The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates median values, whiskers extend to further observation within the I quartile minus 1.5xIQR or within the III quartile plus 1.5xIQR.

Moreover, the increase or decrease of the Δ TNC does not seem to be proportional to the values of the WBC-DIFF counts recorded after mixing blood with supernatants; in approximately one-third of samples, the Δ TNC did not change, despite a marked difference between the WBC-DIFF count of fresh samples and the WBC-DIFF count recorded after mixing blood with the supernatants. This suggests that the final WBC-DIFF count obtained after dilution does not directly influence the Δ TNC. The design of our study does not allow to determine the number of WBCs needed in a feline blood sample to optimize the test. However, the fact that effusions added with the same blood sample often had different Δ TNC values, supports the hypothesis that the Δ TNC is influenced by the type of effusion rather than by the cell count of the added blood. However, it is possible that blood samples with leukopenia, not included in our study, lead to a WBC-DIFF count in the effusion so low as to hamper the possibility of having, in FIP cats, a Δ TNC sufficiently high to

support a clinical diagnosis of FIP. Hence, the use of leukopenic blood samples to spike frozen effusions cannot be recommended based on our results.

In order to assess the effect of dilution, 9 samples (4 from cats with FIP and 5 from cats without FIP) were tested using 2 dilutions of blood: 1:10, as described above, and 1:5 (i.e., 40 μ L of blood in 160 μ L of supernatant). Also in this case, no significant differences were found with the 2 dilutions either in the FIP group or in the non-FIP group, and the classification of samples as consistent or not consistent with FIP did not change at the 1:5 dilution compared with the 1:10 dilution (figure 4).

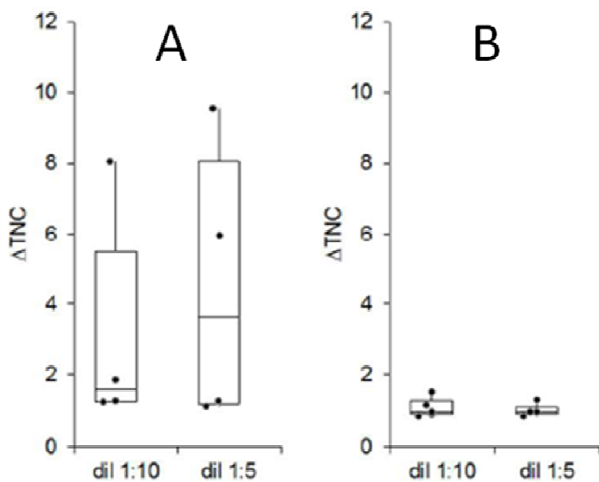


Figure 4. Comparison of the results obtained in frozen-thawed samples with different blood effusion ratios. The Δ TNC recorded in cats with FIP are reported in A, while the Δ TNC recorded in cats without FIP are reported in B.

The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates median values, whiskers extend to further observation within the I quartile minus 1.5xIQR or within the III quartile plus 1.5xIQR.

The 4 FIP effusions were also analyzed using a 1:2 dilution (100 μ L of blood in 100 μ L of supernatant) and, even though no significant differences or changes in the classification of samples were found in this comparison, the Δ TNC decreased in all 4 cases (from 12.6 to 7.7, from 4.10 to 1.22, from 4.75 to 0.74, from 0.98 to 0.97 in a 1:10 and 1:2 dilution, respectively). These data suggest that when the supernatant: blood ratio is low, the amount of fibrin (and other inflammatory proteins) is too low to induce cell clumping given the dilution of proteins responsible for WBC clumping. Therefore, the 1:5 and 1:10 dilution provide similar results, and the 1:10 dilution should be preferred to facilitate the calculation of volumes.

The total protein content of the effusions was high independently on the final diagnosis (2.7 to 6.0 g/dL in non FIP effusions; 3.3 to 7.2 g/dL in FIP effusions) hence it did not influence the Δ TNC values with either the original or the modified method, suggesting that it is not the overall amount of protein that is responsible for the increased Δ TNC, but rather the presence of a particular protein pattern. Using the 1:10 dilution, independently of the differences in Δ TNC values between fresh and frozen–thawed samples, all the effusions from cats without FIP had a Δ TNC <1.7 using

both methods. Conversely, on fresh effusions from cats with FIP, 4 of 14 samples (cats 2, 8, 9, 17) had a Δ TNC <1.7 , 3 of 14 samples (cats 11–13) had a Δ TNC of 1.7 – 3.4 , and 7 of 14 samples (cats 3–7, 14, 15) had a Δ TNC consistent with FIP (>3.4). Using the modified technique, 2 of the 4 “false-negatives” (Δ TNC <1.7 ; cats 2, 8) remained falsely negative, 1 of 4 (cat 17) increased to values suggestive of FIP (Δ TNC of 1.7 – 3.4), and 1 of 4 (cat 9) resulted as positive (Δ TNC >3.4). Only 1 true-positive sample based on the fresh sample (cat 13; which, however, had a Δ TNC of 1.89, close to the lowest cutoff of 1.7) resulted as a false-negative (Δ TNC <1.7) using the modified technique. The 2 other samples that on fresh effusions had a Δ TNC of 1.7 – 3.4 (cats 11, 12) resulted as positive (Δ TNC >3.4) using the modified technique. Therefore, in cats with FIP, using the modified technique, 3 of 19 samples (cats 2, 8, 13) had a Δ TNC <1.7 , 1 of 19 (cat 17) had a Δ TNC of 1.7 – 3.4 , and all the other samples had a Δ TNC >3.4 . Hence, both methods, independently of the cutoff, show absolute specificity and PPV (100.0%). The sensitivity and NPV of the modified method (84.2% and 83.3% at the cutoff of 1.7; 78.9% and 78.9% at the cutoff of 3.4) were higher than that of the original method (78.6% and 81.3% at the cutoff of 1.7; 57.1% and 68.4% at the cutoff of 3.4). Thus, the diagnostic interpretation of Δ TNC using the cutoffs that in study I were considered suggestive of FIP (Δ TNC >1.7) or consistent with FIP (Δ TNC >3.4) indicates that the modified method minimally affects the classification of samples as “FIP” or “non-FIP,” independently of the Δ TNC value obtained from fresh samples. Three samples from cats with FIP had a low Δ TNC count either with both the techniques, probably due to intrinsic properties of the effusions, as noted in the previous study. Thus, if the Δ TNC is negative using both methods, FIP must be confirmed using tests other than the Δ TNC.

The results of our study demonstrated that the modified Δ TNC is an alternative, reliable tool to diagnose effusive FIP in those cases where cytology of the effusion or other biochemical tests on the supernatants cannot be performed immediately after sampling. This method can be performed on frozen supernatants, thus allowing retrospective analyses of stored fluids. Supernatants from FIP effusions, likely because of their protein pattern, retain the ability to clump cells derived from blood samples, whereas the non-FIP effusions do not. Therefore, it may be best to centrifuge effusions that cannot be analyzed immediately, and to remove and send the supernatant to the laboratory. Future studies focused on determining the minimum number of WBCs needed to obtain a positive result may further improve the performance of this alternative method.

III. Frequency of electrophoretic changes consistent with feline infectious peritonitis in two different time periods (2004-2009 vs 2013-2014)

Material and methods

Retrospective search of the database

The database of the DIMEVET laboratory was retrospectively analysed. All the results of serum protein electrophoresis, either data referred to capillary zone electrophoresis (CZE) or data referred to agarose gel electrophoresis (AGE), performed between 2004 and 2014 were downloaded in an Excel sheet for the following selection procedures. Results referred to the two electrophoretic techniques were separately analysed.

Electrophoretic methods and groups formation

Agarose gel electrophoresis and capillary zone electrophoresis were performed using automated systems and kits provided by the manufacturer of the instruments, Hydrasis and Minicap respectively (Sebia Italia Srl, Bagno a Ripoli, Florence, Italy). For AGE, after 10 µl of each serum sample were manually loaded in the applicator, a 0.8% agarose gel was run in trisbarbital buffer at pH 8.6, with a migration time of 7 mins at 20 W Gels were then dried (10 mins at 65°C), automatically stained with Amido Schwarz, destained and dried again for scanning by the appropriate gel scanner. For CZE, serum samples were aspirated from the anode end of a silica capillary; proteins were then separated at 35°C by migration for 2.5 mins at high voltage (9000 V) in an alkaline buffer (pH 9.9). For AGE, data were then transferred to the software (Phoresis, Sebia Italia Srl) which analyses the density of each band, creating a corresponding peak, and separates each fraction based on the slopes of the curves. For CZE, variations of absorbance due to the protein flow were read through a spectrophotometer at 240 nm wavelength and recorded in real time by the software program (Phoresis) producing the typical electrophoretic peaks.

For both the techniques, albumin, α_1 -, α_2 -, β_1 -, β_2 - and γ -globulin fractions were generated. For the purpose of this study, only data regarding electrophoretic fractions important for the diagnosis of FIP (total protein, albumin, α_2 -, and γ -globulin, total globulin and A/G ratio) were recorded and statistically analyzed. Absolute protein concentrations (g/dL) for each electrophoretic fraction were calculated based on the percentage of the area under each peak and on total serum protein

concentrations measured through an automated spectrophotometer (Cobas Mira, Roche) using the biuret method, except for 6 cases (5 processed with AGE and 1 with CZE), on which total proteins were not measured. In these cases, only the percentages of the different fractions were included in the statistical analysis.

Data regarding SPE were divided in two groups:

FIP: cats with necropsy and histology consistent with FIP and with intralesional coronaviruses detected by immunohistochemistry,

Healthy cats: samples included in this group were submitted for annual wellness visits or for preoperative examinations or were included as a control group in previous studies and had unremarkable clinical or laboratory findings (Paltrinieri et al., 2014a; Paltrinieri et al., 2014b).

Statistical analysis

Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it v-2.1 software (Analyse-it Software Ltd, Leeds, UK).

Results obtained in the two time spans (2004-2009 vs 2013-2014) from FIP and healthy cats were compared to each other using a nonparametric *t*-test for independent measurements (*Mann Whitney U test*). The significance level was set at $p < 0.05$.

For the two time periods examined in this study, the number of cats from the FIP group showing single or combined electrophoretic changes consistent with FIP was counted (Pedersen, 2009; Giordano and Paltrinieri, 2010). To this aim, the relative and absolute value of the electrophoretic parameters listed above (expressed as percentage and g/L respectively) were compared with the reference intervals described in a previous study for AGE and CZE, respectively (Giordano and Paltrinieri, 2010).

In addition, each electrophoretogram obtained from FIP cats underwent a visual analysis performed by two operators in a blind manner. Electrophoretograms were interpreted as “consistent with FIP” if showing both α_2 - and γ -globulin polyclonal peaks; as “dubious” if showing only one of the mentioned peaks and as “not consistent with FIP” if α_2 - and γ -globulin polyclonal peaks were both absent or if alterations typical of other diseases (e.g. monoclonal gammopathy) were detected (figure 1). In the case of disagreement between the classification of the electrophoretograms by the two observers, each electrophoretogram was evaluated as a man to reach a final consensual interpretation.

Both the comparison with reference intervals and the visual analysis were used to calculate the number of FIP cats with electrophoretic pattern consistent with FIP in order to calculate the sensitivity of the method.

A Pearson chi-square analysis was used to evaluate the possible differences in the proportion of changes consistent with FIP either in terms of relative or absolute changes compared with the reference values and in terms of visual analysis.

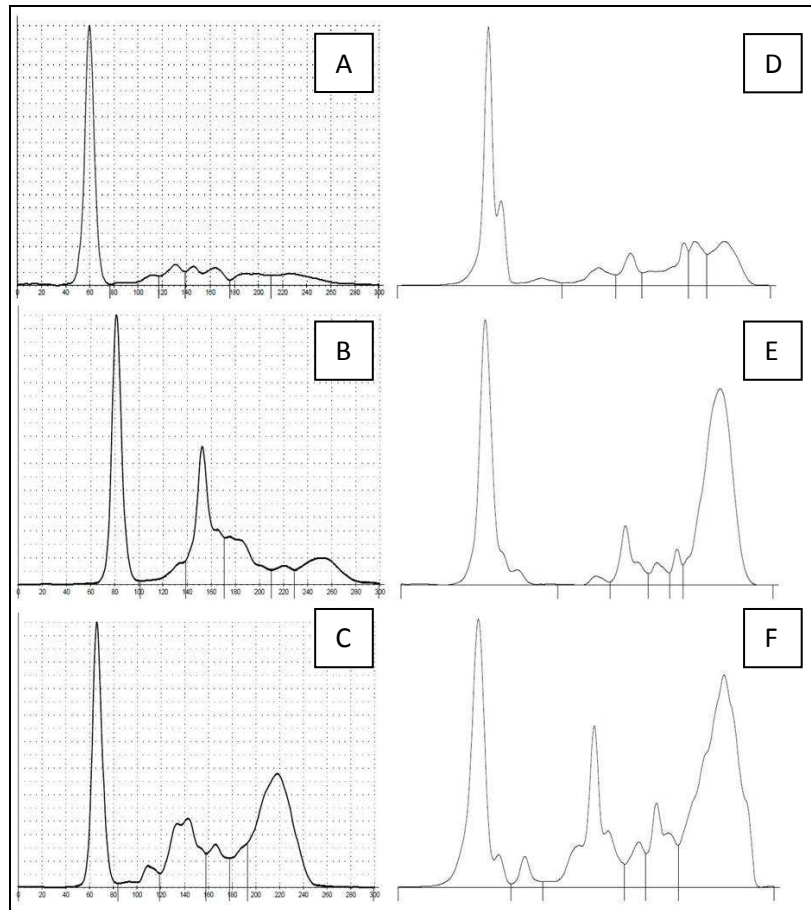


Figure 1. Examples of electrophoretograms considered (A, D) not consistent with feline infectious peritonitis (FIP), (B, E) dubiously consistent with FIP and (C, F) consistent with FIP, performed with (A–C) agarose gel electrophoresis and (D–F) capillary zone electrophoresis. A, D: no electrophoretic abnormality; B: α 2-globulin but not γ -globulin increased; E: increased γ -globulin but not α 2-globulin; C, F: both α 2- and γ -globulin are increased.

Results

Retrospective search and final caseload

The following selection procedure, reported in figure 2, led to a final caseload of 91 AGE and 45 CZE. A similar proportion of effusive and non effusive forms was found in the two study period either for AGE or for CZE (dry forms = 3/17 [17.6%] in the first period; 5/24

[20.8%] in the second period) or for CZE (2/10 [20.0%] in the first period; 4/16 [25.0%] in the second period).

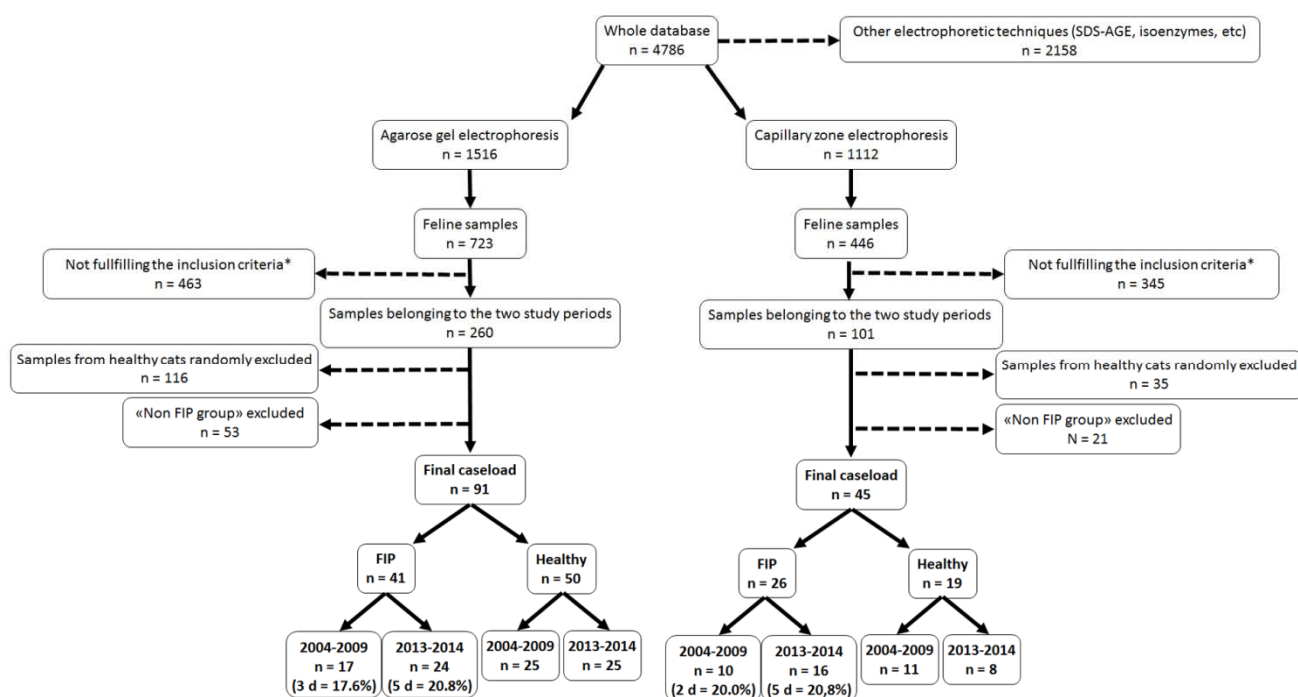


Figure 2. Flow diagram summarising the selection of cases from the database. Samples from healthy cats were randomly excluded in order to level out the FIP and the NON FIP cats groups.

*Absence of a final diagnosis; samples collected outside the study period (2004–09 or 2013–14); wrong/difficult separation of fractions. SDS-AGE= sodium dodecyl sulfate agarose gel electrophoresis. d= numbers of dry forms recorded in each period.

Comparison between results obtained in the two study periods in healthy cats and in cats with FIP

Results about the comparison of each electrophoretic fraction obtained in the two study periods are reported in table 1. Using AGE, no significant differences between the study periods were found in healthy cats. Conversely, in cats with FIP, the concentration of total protein and the concentration and the percentage of total- and γ -globulin were significantly lower, while the percentage of albumin, α_2 -globulins and the A/G ratio were significantly higher in recent years (2013-2014) compared with the former period (2004-2009). Using CZE, no significant differences were found between the two study periods, either in cats with FIP or in clinically healthy cats. The absence of statistically significant differences between healthy cats in the two study periods demonstrates that any possible difference found in FIP cats over time are unlikely imputable to changes in the analytical performance of the technique.

	Unit	AGE				CZE			
		FIP		Healthy		FIP		Healthy	
		2004-2009 (n=17)*	2013-2014 (n=24)*	2004-2009 (n=25)	2013-2014 (n=25)	2004-2009 (n=10)‡	2013-2014 (n=16)	2004-2009 (n=10)	2013-2014 (n=9)
Total protein	g/dL	9,1 ± 1,5 (9,1) 5,8-12,1	8,1 ± 1,7§ (8,8) 5,3-11,6	6,98 ± 0,68 (7,03) 5,55-8,11	6,98 ± 0,83 (6,75) 5,51-8,20	8,2 ± 1,4 (8,4) 5,8-9,9	7,9 ± 1,7 (7,9) 5,1-11,5	6,8 ± 0,5 (7,0) 5,7-7,5	6,5 ± 0,9 (6,8) 4,4-7,3
		29,1 ± 14,0 (27,9) 7,6-58,6	37,9 ± 10,7¶ (38,1) 14,0-54,2	54,10 ± 7,65 (57,30) 37,70-64,70	52,34 ± 7,20 (51,50) 41,10-68,00	30,0 ± 9,0 (27,9) 18,2-50,4	32,4 ± 8,2 (32,4) 12,7-46,0	55,2 ± 5,2 (57,4) 44,4-61,4	54,6 ± 4,7 (53,2) 49,2-62,2
		2,89 ± 1,36 (3,31) 0,70-5,81	3,03 ± 1,03 (2,70) 1,56-5,04	3,77 ± 0,63 (3,75) 2,37-5,11	3,63 ± 0,53 (3,54) 2,76-4,97	2,60 ± 1,12 (2,45) 1,05-4,99	2,48 ± 0,51 (2,56) 1,36-3,22	3,76 ± 0,33 (3,71) 3,20-4,37	3,54 ± 0,56 (3,68) 2,19-4,08
α ₂ -globulin	%	10,6 ± 6,8 (8,2) 2,5-28,8	17,2 ± 7,3∞ (16,6) 6,7-33,3	12,7 ± 3,33 (13,4) 6,4-18,9	14,3 ± 2,8 (14,3) 8,8-18,8	17,1 ± 6,4 (17,4) 7,8-27,3	16,7 ± 5,2 (16,2) 7,5-25,9	14,6 ± 3,0 (15,6) 8,4-18,2	12,7 ± 2,2 (12,9) 9,5-16,0
		0,97 ± 0,67 (0,81) 0,22-2,65	1,30 ± 0,57 (1,22) 0,59-3,25	0,89 ± 0,24 (0,91) 0,42-1,24	1,00 ± 0,22 (0,98) 0,53-1,33	1,45 ± 0,57 (1,45) 0,45-2,47	1,27 ± 0,26 (1,33) 0,67-1,67	0,99 ± 0,18 (1,00) 0,63-1,24	0,83 ± 0,21 (0,88) 0,55-1,09
		41,6 ± 17,4 (44,6) 13,5-72,7	27,0 ± 16,2¶ (28,5) 5,7-68,3	16,81 ± 4,31 (16,00) 7,90-25,50	17,37 ± 4,82 (17,60) 8,80-27,70	34,9 ± 16,0 (34,5) 13,1-61,2	32,5 ± 14,8 (32,3) 6,6-59,1	17,9 ± 8,0 (18,6) 6,0-31,1	20,8 ± 5,1 (21,7) 10,6-26,8
γ-globulin	g/dL	3,57 ± 1,67 (3,43) 1,24-6,58	2,43 ± 1,80¶ (2,07) 0,35-7,92	1,17 ± 0,33 (1,10) 0,59-1,87	1,23 ± 0,42 (1,10) 0,52-2,21	2,62 ± 1,14 (2,82) 1,21-4,71	2,78 ± 1,65 (2,49) 0,34-6,32	1,24 ± 0,61 (1,23) 0,43-2,24	1,34 ± 0,38 (1,48) 0,72-1,85
		70,9 ± 14,0 (72,1) 41,4-92,4	62,1 ± 10,7¶ (61,9) 45,8-86,0	45,90 ± 7,65 (42,70) 35,30-62,30	47,66 ± 7,20 (48,50) 32,00-58,90	70,0 ± 9,0 (72,0) 49,6-81,8	67,6 ± 8,2 (62,8) 54,0-87,3	44,8 ± 5,2 (42,6) 38,6-55,6	45,4 ± 4,7 (46,8) 37,8-50,8
		6,26 ± 1,69 (6,04) 4,10-8,88	5,10 ± 1,70¶ (4,31) 2,89-9,98	3,21 ± 0,64 (3,10) 2,08-4,48	3,35 ± 0,75 (3,09) 2,05-4,83	5,61 ± 0,81 (5,82) 4,42-6,98	5,47 ± 1,76 (5,16) 2,93-9,34	3,07 ± 0,53 (2,84) 2,28-4,01	2,94 ± 0,52 (3,22) 2,24-3,52
A/G ratio		0,47 ± 0,35 (0,39) 0,08-1,42	0,66 ± 0,29¶ (0,62) 0,16-1,18	1,24 ± 0,36 (1,34) 0,61-1,83	1,15 ± 0,36 (1,06) 0,70-2,13	0,45 ± 0,23 (0,39) 0,22-1,02	0,50 ± 0,18 (0,48) 0,15-0,85	1,25 ± 0,25 (1,35) 0,80-1,59	1,23 ± 0,24 (1,14) 0,97-1,65

Table 1. Comparison of each electrophoretic fraction between the two study periods in cats with FIP and healthy cats analysed with agarose gel (AGE) and capillary zone (CZE) electrophoresis. Results are presented as mean ± SD, median (in brackets) and I-III interquartile intervals

*n = 14 for values in g/dl; †n = 22 for values in g/dl; ‡n = 9 for values in g/dl;

§P < 0.001 vs 2004-2009; ¶P < 0.05 vs 2004-2009 ∞P < 0.01 vs 2004-2009

Frequency of electrophoretic alterations in comparison with reference intervals

In the first period (2004-2009) all the cats with FIP tested with AGE and most of the cats with FIP tested with CZE had hyperproteinemia (table 2), while the proportion of cats with FIP and hyperproteinemia was lower in the second period, with a significant difference for AGE. Independently on the electrophoretic technique, the proportion of FIP cats with a simultaneous increase of α_2 - and γ -globulin was similar in the two study periods, while in the second period a higher rate of cases characterized by an increase of α_2 -globulins without gammopathy and a lower rate of cases characterized by gammopathy but not by the increases of α_2 -globulin were found and, in this latter case, a significant difference was found for percentage values in samples processed by AGE, and for absolute values in samples processed with both the techniques.

	UNIT	AGE		CZE	
		2004-2009	2013-2014	2004-2009	2013-2014
Hyperproteinaemia with low A:G ratio	g/dl	14/14 (100,0)	14/22 (63,6)*	7/9 (77,8)	9/16 (56,3)
Increased α_2 -globulin but normal γ -globulin	%	1/17 (5,9)	3/24 (12,5)	0/10 (0,0)	3/16 (18,8)
Increased γ -globulin but normal α_2 -globulin	%	13/17 (76,5)	10/24 (41,7)*	6/10 (60,0)	7/16 (43,8)
Increased α_2 - and γ -globulin	%	0/17 (0,0)	2/24 (8,3)	3/10 (30,0)	6/16 (37,5)
Increased α_2 -globulin but normal γ -globulin	g/dl	2/14 (14,3)	6/22 (27,3)	0/9 (0,0)	3/16 (18,8)
Increased γ -globulin but normal α_2 -globulin	g/dl	10/14 (71,4)	7/22 (31,8)*	2/9 (22,2)	0/16 (0,0)*
Increased α_2 - and γ -globulin	g/dl	2/14 (14,3)	4/22 (18,2)	7/9 (77,8)	13/16 (81,3)

Table 2. Frequency of electrophoretic alterations compared with the reference intervals. Results are presented as number of observations/total number of cases (%) * $P < 0.05$ vs 2004–2009

Visual interpretation of electrophoretograms in the two time spans

The agreement between the two operators resulted absolute (100%) for AGE. Using AGE, only half of the FIP cats showed the typical pattern of FIP, while this proportion increased using CZE, confirming that the sensitivity of this test is not absolute (table 3). Anyway, the diagnostic sensitivity of SPE resulted similar in the two examined time spans, while the number of the dubious patterns increased in the second period with AGE but not with CZE. However, all these differences were not statistically significant.

	AGE		CZE	
	2004-2009	2013-2014	2004-2009	2013-2014
Consistent with FIP	9/17 (52.9)	13/24 (54.2)	6/10 (60.0)	12/16 (75.0)
Dubious	4/17 (23.5)	10/24 (41.7)	3/10 (30.0)	4/16 (25.0)
Not consistent with FIP	4/17 (23.5)	1/24 (4.1)	1/10 (10.0)	0/16 (0.0)

Table 3. Results of the visual interpretation analysis performed by two operators on the electrophoretograms of cats with feline infectious peritonitis (FIP). Results are presented as number of observations/total number of cases (%).

Discussion

In this study, the performance of SPE was retrospectively studied in order to understand if electrophoretic patterns not consistent with FIP, anecdotally observed in recent years in our laboratory in FIP cats, were really more frequent than in the past.

In the FIP group, the percentage of effusive and non effusive forms was similar for the two electrophoretic techniques examined in this study and the proportion of samples retrospectively analysed after freezing and analysed with the two techniques was similar in the two periods, both demonstrating that changes in the electrophoresis patterns are unlikely associated with a different rate of effusive vs non effusive FIP and excluding that differences in the patterns could be associated with storage artefacts, which are anyway known to minimally affect SPE (Eckersall, 2008; Stockham and Scott, 2008). Regarding FIP cats tested with AGE, total protein concentration was significantly lower in the recent period in comparison with the previous one. In addition, total protein concentrations in the recent period were frequently close to or lower than the reference intervals established in our laboratory. Both the relative and the absolute concentrations of total and γ -globulins were significantly lower in the second period. Therefore, the albumin/globulin ratio was significantly higher and frequently within the reference interval in FIP cats in the second period, in contrast with what reported by Sparkes et al. (1991), who recorded a high prevalence of low albumin/globulin ratio in FIP cats.

The analysis of results obtained with CZE revealed the same trends over time either for total proteins and for electrophoretic fractions, but the differences were not statistically significant. Also, the comparison with the reference intervals in the two periods confirmed that the proportion of electrophoretic alterations in FIP cats for both the electrophoretic techniques was higher in the first period, also in this case with different statistical significance between AGE and CZE, likely due to the different numbers of samples in the caseload. While all the FIP cats analysed

with AGE and most of the cats analysed with CZE presented hyperproteinemia in the first period, this alteration was less frequent in the recent years. The most frequent difference between the two periods was the decreased frequency of hypergammaglobulinemia, which is known to be the most typical finding in FIP cats (Pedersen, 2009). Another interesting aspect was the increased percentage, in the second period and for both the techniques, of increased α_2 -globulins not associated with a concurrent gammopathy. Theoretically, the absence of the gammopathy may be imputable to the time of sampling since experimental studies showed that γ -globulins increase a couple of weeks later than the increased of α_2 -globulins (Addie et al., 2004). In the current study, in two cats with increased α_2 -globulin and normal γ -globulins tested in the first period of time (one with AGE and one with CZE), the gammopathy appeared when electrophoresis was repeated two weeks later. Conversely, three cats with the same electrophoretic profile tested in the second period (two with AGE and one with CZE) did not develop the gammopathy in samplings repeated in the following month. This suggests that in the second period of the study the high frequency of increased α_2 -globulin in the absence of gammopathy may not depend on the stage of the disease. The results of this study seem to indicate a less intense antigenic stimulation in FIP cats, reflected by γ -globulin concentrations lower than in the past and closer to the reference intervals of our laboratory. This finding is likely related to a lower concentration of immunoglobulins which are the most relevant proteins known to migrate in the gamma region (Stockham and Scott, 2008). A possible explanation for this finding is a variation of the host-coronavirus interactions, possibly due to changes in viral immunogenicity and/or to the development of a different immune response of the feline population analysed. The design of this study does not allow to investigate this pathogenic aspect, but our results are encouraging to design future studies on the characteristics of viral populations recently isolated or on host-virus interactions. Conversely, results on repeated samplings above mentioned tend to exclude the possibility that the differences reported over time depend on an earlier identification of symptoms as compatible with FIP by the referring veterinarians and consequently on the analysis of samples collected in an early stage of disease, when the antigenic response has not yet been triggered. Also, the possible effect of drug administration on the development of gammopathy seems unlikely since samples were submitted before the provisional diagnosis was emitted and therefore before starting any treatment. Even if the possible reasons for these changes were not investigated in this study, it is important to highlight how the absence of the typical electrophoretic alterations could affect the

interpretation of electrophoretograms and the diagnosis of FIP, especially in its non effusive form, since in this latter form tests on effusions (e. g. immunofluorescence, Rivalta's test), which are known to be accurate, are not available (Hartmann, 2005; Fischer et al., 2012). Regarding this aspect, the visual interpretation performed on the electrophoretic patterns pointed out the low sensitivity of this approach for the diagnosis of FIP and also how the "dubious patterns" (e.g. the presence of one of the typical alterations for FIP alone) increased in the second period for AGE. The results obtained in this study with AGE were slightly discordant with those obtained with CZE, likely due to the higher resolution typical of this latter technique that allows to detect higher and narrower peaks, that may thus become visually evident also when percentage or absolute values are closer to or lower than the upper reference limits (Giordano and Paltrinieri, 2010). This information suggests that CZE, probably due to its analytical properties like its higher resolution compared with AGE (Giordano and Paltrinieri, 2010), may have a better sensitivity compared to AGE, especially in identifying the gammopathy, which seems to be less frequent in FIP cats in the recent time span.

IV. Comparison of the diagnostic performances of laboratory tests for the diagnosis of feline infectious peritonitis

Material and methods

Caseload and laboratory methods

This was a retrospective study based on the results recorded with different clinico-pathological or molecular methods on samples submitted to the diagnostic laboratory of the DIMEVET laboratory between 2013 to 2015. The laboratory database was analysed to select data fulfilling the following criteria:

- Clinical suspicion of FIP, based on history and clinical signs (fever, lethargy, anorexia, weight loss, neurological/ocular signs, effusions, etc.)
- Availability of a final diagnosis, based on post-mortem findings or on 18 months follow up, as specified below
- Presence of at least one result regarding the clinico-pathological tests (serum protein electrophoresis and α_1 -acid glycoprotein measurement on blood, cytology and Δ TNC measurement on effusions) or the molecular tests (nRT-PCR for 3'UTR and Spike gene sequencing on blood, effusions and tissues) included in this study.

All the samples were analysed for routine diagnostic purposes and collected under informed consent of the owners, therefore a formal approval from the Ethical Committee was not required (EC decision 29 Oct 2012, renewed with the protocol n° 02-2016).

Cats were assigned to the FIP group if typical histopathological findings along with a positive result on immunohistochemistry against the FCoV in at least one tissue were present (Figure 1) (Pedersen, 2009). Cats were assigned to the non-FIP group when histology revealed the presence of diseases other than FIP along with negative results on immunohistochemistry on all the sampled tissues, when the follow-up demonstrated a complete recovery (remission of the symptoms, normalization of the recorded laboratory alterations) after 18 months from the first diagnostic procedures, or when a definitive diagnosis was achieved through collateral laboratory and/or imaging analyses.

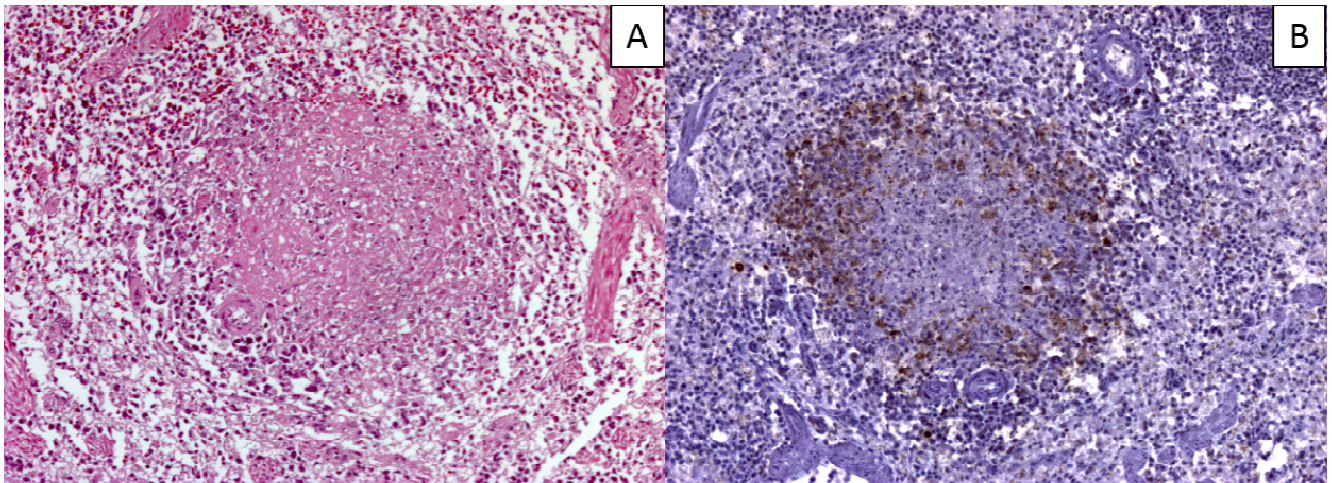


Figure 1. Example of a positive histology and immunohistochemistry for the assignment of the cats to the FIP group. A. Haematoxylin-eosin stained section of a spleen showing a typical FIP lesion, with a necrotic center surrounded by neutrophils and macrophages. B. The same section with positive result at immunohistochemical examination, with the surrounding macrophages containing the viral antigen stained in golden/brown.

Post-mortem examinations

Cats euthanized by the referring veterinarians due to the worsening of the clinical conditions were kept refrigerated until subjected to necropsy within 6 hours. One specimen from all organs affected by gross lesions was collected, fixed in 10% buffered formalin and embedded in paraffin. Another specimen from at least one affected organ (mostly mesenteric lymph node) was immediately frozen at -80°C for molecular analyses.

Histology was performed on microtomic sections ($5\ \mu\text{m}$) stained with haematoxylin-eosin.

Immunohistochemistry was performed using a mouse monoclonal antibody anti-FCoV (FIPV3-70 clone, Serotec) and the standard avidin biotin complex (ABC- Vectastain Elite, Vector laboratories Inc.) method (Hsu et al., 1981). As a chromogen, 3,3'-Diaminobenzidine (DAB) (Vector Laboratories) was used. The slides were counterstained with Mayer's haematoxylin. In each working session, a section of kidney from a cat with confirmed FIP served as positive control. Negative controls consisted of serial sections of tested tissues in which anti-FCoV antibody was replaced with normal not immune mouse serum (DAKO; Glostrup Denmark).

On FIP cats, immunohistochemistry was performed on selected specimens with typical histological lesions, while in non-FIP cats all the tissues collected during necropsy were tested with immunohistochemistry, in order to definitively exclude the presence of FCoVs.

Tests on serum

Total proteins were spectrophotometrically measured using the biuret method (Cobas Mira, Roche), then SPE was performed on agarose gel using the automated analyser Hydrasis (Sebia Italia Srl) and a specific kit (Hydragel 15 β 1- β 2, Sebia Italy Srl) as previously described (Giordano and Paltrinieri, 2010). Gels were scanned and data were analysed with the appropriate software (Phoresis, Sebia Italia Srl).

The serum concentration of AGP was measured using a radial immunodiffusion kit (SRID, Tridelta Development Limited) as previously described (Paltrinieri et al., 2007).

Tests on effusions

Fresh effusions were analysed with the Sysmex XT-2000iV (Sysmex Europe GmbH, Norderstedt, Germany). The Δ TNC was recorded as reported in study I (see above).

Cytological examination was performed by an ECVCP diplomate on smears or on cytocentrifuged samples stained with a rapid stain (Hemacolor, Merck).

Molecular tests on blood, effusions and tissues

Whole blood and effusions collected in EDTA were centrifuged ($3,000 \times g$, 5 min) within 12 hours and the obtained pellets were suspended in 200 μ L of PBS by vigorous vortexing and immediately stored at -20°C for further RNA extraction.

Samples were subjected to RNA extraction using a NucleoSpin RNA commercial kit (Macherey-Nagel), according to the manufacturer's instruction. The extracted RNA was screened for the presence of FCoV using a RT-nPCR targeting a 177 bp product of the highly conserved 3' untranslated region (3' UTR) of the genome of both type I and type II FCoV (Herrewegh et al., 1995b). FCoV RNA was used as positive control and RNase-free water as negative control. PCR products were visualized under UV transilluminator on a 2% agarose gel stained with ethidium bromide. The extracted RNA subjected to S gene sequencing was previously tested using an RT-nPCR targeting a 142 bp product of the S gene (Chang et al., 2012). Positive samples were sequenced using the forward and reverse primers used for the second reaction. Sequencing was performed at an external laboratory (Parco Tecnologico Padano, Lodi, Italy) using a Big Dye Terminator version 3.1 Cycle Sequencing kit and an AB3730 DNA Analyzer (Applied Biosystems).

Sequence data were assembled and manually corrected using BioEdit software version 7.0¹. Consensus sequences were aligned with FCoV strains bearing or not the mutations M1058L or S1060A, retrieved from GenBank, using Clustal X in the BioEdit software.

Calculation of diagnostic performances

Samples were classified as positive when features considered as consistent with FIP based on literature (table 1) were present, and as negative (not consistent with FIP) when findings reported in table 1 were absent or when features considered as dubious or suggestive (but not confirmatory) for FIP were present. Dubious features included the lack of granular background in cytological samples, Δ TNC values between 1.7 and 3.4 (the cut-off values established in study I), increased serum α_2 -globulins but normal γ -globulins or vice-versa, or AGP values between 0.56 (reference value of the laboratory) and 1.5 mg/mL (figure 2) (Paltrinieri et al., 2007).

When molecular tests were performed on more than one tissue specimen, cats were considered as positive when at least one of the tested organs provided a positive result and negative when all the tested organs resulted negative.

For each test, TP and FP results (results consistent with FIP in cats with and without FIP, respectively), TN and FN results (results not consistent with FIP in cats without and with FIP, respectively) were recorded. Sensitivity, specificity, as well as positive and negative likelihood ratios (LR+ and LR-) were then calculated (Christenson, 2007).

¹ See <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>

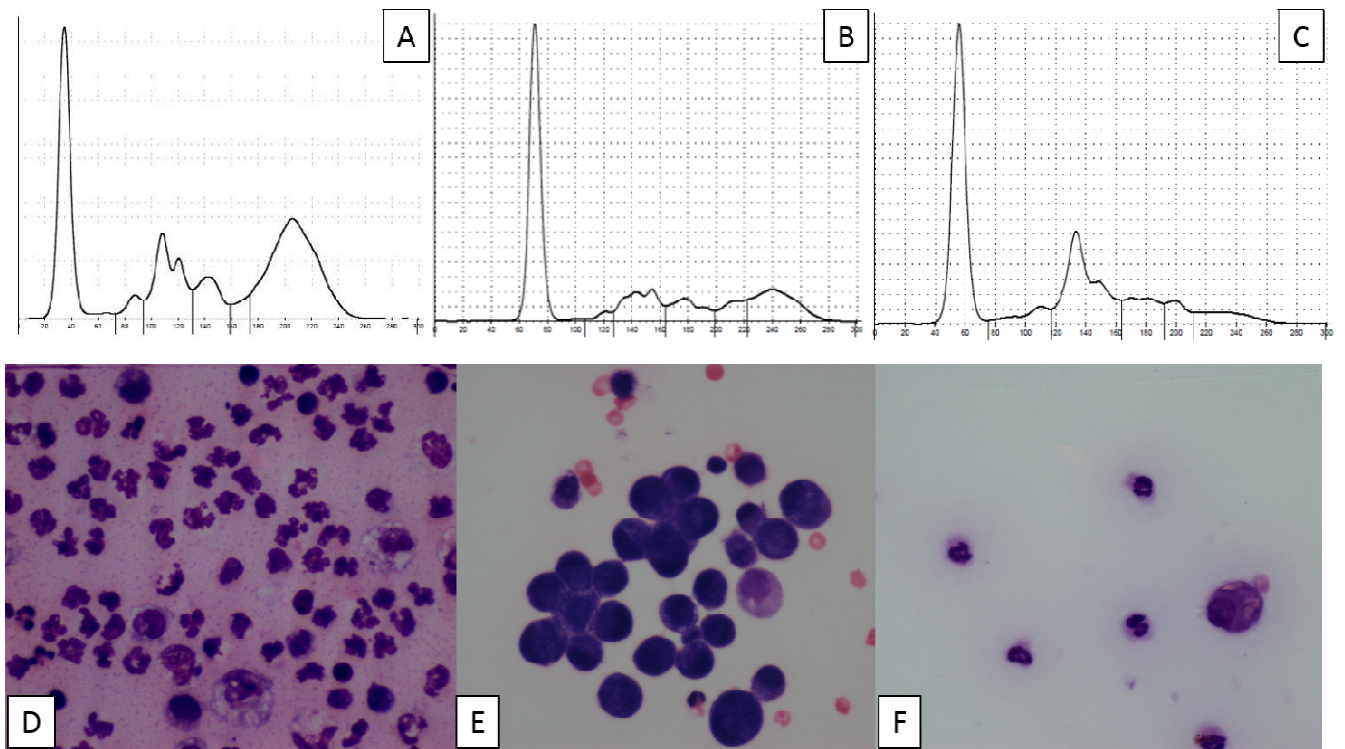


Figure 2. Serum protein electrophoretic and cytological patterns used for the calculation of diagnostic performances. A: SPE consistent with FIP (increased α_2 - and γ globulin with a polyclonal peak); B: SPE not consistent with FIP (absence of α_2 - and γ globulin with a polyclonal peak); C: SPE dubious for FIP (presence of α_2 -globulin peak only); D: cytological pattern consistent with FIP (presence of an inflammatory non-specific process and of a proteinaceous background); E: cytological pattern not consistent with FIP (adenocarcinoma), F: cytological pattern dubious for FIP (presence of an inflammatory non-specific process without the proteinaceous background).

Specimen	Test	Features and cut-offs consistent with FIP
Effusion	Cytology	Presence of an inflammatory non-specific process and of a proteinaceous background
	Δ TNC	>3.4
	RT-nPCR 3' UTR	Positive result
	S gene sequencing	Presence of M1058L or S1060A mutations
Blood	SPE	Increased α_2 - and γ globulin with a polyclonal peak
	AGP	>1.5 mg/mL
	RT-nPCR 3' UTR	Positive result
	S gene sequencing	Presence of M1058L or S1060A mutations
Tissues	RT-nPCR 3' UTR	Positive result
	S gene sequencing	Presence of M1058L or S1060A mutations

Table 1. Laboratory tests performed on the different specimens and features and/or cut-offs that were considered consistent with FIP (Herrewegh et al., 1995b; Sparkes et al., 1991; Paltrinieri et al., 2001; Paltrinieri et al., 2007; Pedersen, 2009; Chang et al, 2012).

Results

Thirty cats (age range: four months to 13 years) suspected to have FIP because of their history and clinical signs were included in this study. The FIP group included 16 cats on which FIP was confirmed post-mortem through histology and positive immunohistochemistry on at least one examined tissue.

The non-FIP group included 14 cats. In three of these, FIP was ruled out based on normalization of clinical and laboratory findings during a one-year follow-up (cats n° 20, 21 and 22, with persistent fever, inappetence and lethargy); in three cats a disease other than FIP was diagnosed through cytology, imaging and flow cytometry (cats n° 24 with hepatic carcinoma and cats n° 28 and 29, both with lymphoma); in 8 cats post-mortem findings were consistent with a disease other than FIP and no FCoV were detected on tissues by immunohistochemistry (cats n° 17, 18, 19, 23, 25, 26, 27 and 30 with renal failure, pleuro-pericardial fibrosis, pleomorphic sarcoma, lymphocytic cholangitis, intestinal carcinoma, myelofibrosis, polycystic kidney disease and lymphoma, respectively).

The samples collected from each case are summarized in table 2, along with the results obtained in the different tests performed on blood, effusions or tissues. Sensitivity, specificity, and likelihood ratios of each test are reported in table 3.

On blood, all the tests had a high or absolute specificity and a high LR+, while the sensitivity was low, except for the measurement of AGP. This test had the highest sensitivity and LR+ and the lowest LR-. SPE and S gene sequencing had the highest LR- values.

On effusions, all the tests had high to absolute sensitivity and specificity and, consequently, high to absolute LR+ and low to excellent LR-, except for the S gene sequencing that had the worst performance in terms of sensitivity LR+ and LR-.

On tissues, the RT-nPCR 3'UTR had the best sensitivity and a low LR-, but also low specificity and low LR+. Conversely, S gene sequencing had a high specificity and LR+ but a lower sensitivity and a slightly higher LR-.

Specimen	Test	Sens	Spec	LR+	LR-
Blood	SPE	42.9%	90.0%	4.29	0.63
	AGP	85.7%	81.8%	4.71	0.17
	3' UTR PCR	75.0%	100.0%	nc	0.25
	S gene sequencing	42.9%	100.0%	nc	0.57
Effusions	Cytology	100.0%	83.3%	6.00	0.00
	Δ TNC	80.0%	100.0%	nc	0.20
	3' UTR PCR	100.0%	83.3%	6.00	0.00
	S gene sequencing	40.0%	83.3%	2.40	0.72
Tissues	3' UTR PCR	90.9%	50.0%	1.82	0.18
	S gene sequencing	70.0%	87.5%	5.60	0.34

Table 3. Sensitivity (Sens), Specificity (Spec), positive and negative likelihood ratio (LR+ and LR-, respectively) calculated for each test and specimens.

Δ TNC: ratio between total nucleated cells counted on the two channels of the Sysmex XT-2000iV; SPE= serum protein electrophoresis; AGP= α_1 -acid glycoprotein; nc= not calculable based on the 100% specificity

Discussion

This study was designed to compare the diagnostic performances of some of the tests currently employed for the diagnosis of FIP since only few studies compared the diagnostic accuracy of different tests (Sparkes and others 1991, Paltrinieri and others 2001, Hartmann and others 2003, Giori and others 2011). Strict inclusion criteria (i.e. only cases with a conclusive diagnosis or with a complete recovery during the follow up were included in the study) were applied in order to increase the reliability of the results, but inevitably reducing the caseload. To minimize the negative effect of the small number of cases (and especially of “non-FIP cats”), the informative power of each test was calculated in terms of likelihood ratios that, contrarily to predictive values, are not influenced by the prevalence of the disease and may provide information about the tests that may increase or decrease the post-test probability of FIP (LR+ notably >1.0 or LR- close to 0.0, respectively).

Comparison of the diagnostic performances of laboratory tests for the diagnosis of feline infectious peritonitis

Group	ID	Blood				Effusion				Tissue	
		EF	AGP	RT-nPCR 3'UTR	S gene seq	Cytology	ΔTNC	RT-nPCR 3'UTR	S gene seq	RT- nPCR 3'UTR	S gene seq
FIP	1	NEG	NEG	-	-	-	-	-	-	-	-
	2*	POS	POS	POS	NEG	POS	NEG	POS	NEG	POS	NEG
	3	NEG	POS	POS	POS	-	-	-	-	POS	POS
	4*	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS
	5	NEG	NEG	NEG	NEG	-	-	-	-	POS	POS
	6*	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	7*	NEG	POS	POS	NEG	POS	POS	POS	NEG	-	-
	8*	NEG	POS	NEG	-	POS	POS	POS	POS	POS	POS
	9	POS	POS	-	-	-	-	-	-	-	-
	10*	NEG	POS	POS	NEG	-	POS	POS	NEG	POS	POS
	11*	NEG	POS	-	-	POS	POS	POS	NEG	POS	NEG
	12*	POS	POS	-	-	POS	POS	POS	NEG	-	-
	13*	POS	POS	-	-	-	POS	POS	POS	POS	POS
	14*	-	-	-	-	-	NEG	POS	NEG	POS	NEG
	15	-	-	-	-	-	-	-	-	NEG	-
	16	POS	POS	-	-	-	-	-	-	-	-
Total positive results		6/14	12/14	6/8	3/7	7/7	8/10	10/10	4/10	10/11	7/10
Non FIP	17	-	NEG	-	-	-	-	-	-	POS	NEG
	18*	NEG	POS	-	-	NEG	NEG	NEG	NEG	NEG	NEG
	19*	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG
	20	NEG	NEG	NEG	NEG	-	-	-	-	-	-
	21	NEG	NEG	NEG	-	-	-	-	-	-	-
	22	NEG	NEG	-	-	-	-	-	-	-	-
	23	POS	POS	NEG	NEG	-	-	-	-	POS	POS
	24*	-	-	-	-	NEG	NEG	NEG	NEG	-	-
	25*	NEG	NEG	-	-	POS	NEG	POS	POS	NEG	NEG
	26	-	-	NEG	NEG	-	-	-	-	NEG	NEG
	27	-	-	-	-	-	-	-	-	POS	NEG
	28*	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	-	-
	29*	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	-	-
	30	NEG	NEG	NEG	NEG	-	-	-	-	NEG	NEG
Total positive results		1/10	2/11	0/8	0/7	1/6	0/6	1/6	1/6	4/8	1/8

Table 2. Specimens collected from each cat and results of the different tests performed on blood, effusions or tissue.* Presence of effusion; ΔTNC: ratio between total nucleated cells counted on the two channels of the Sysmex XT-2000iV; SPE= serum protein electrophoresis; AGP= α₁-acid glycoprotein; - = not performed

In this study, in fact, the prevalence of FIP was higher than in similar previous reports that compared the diagnostic performances of different tests (Beatty and Barrs, 2010), since the number of cats with signs consistent with FIP but affected by other disease was low. Additionally, dubious results were considered as negative, since they cannot confirm the disease when taken alone. Moreover, the low rate of inflammatory conditions in the non-FIP group may have overestimated the specificity of tests suggestive of inflammation (cytology of the effusion, SPE, AGP measurement). Despite these limitations, this study confirmed that none of the tests examined in this study can be used alone to diagnose FIP.

In blood, SPE had a very low sensitivity since FIP cats showed dubious or negative patterns, in disagreement with previous reports, and the LR ratios indicate that SPE cannot definitely rule out FIP but it may be a confirmatory test (Sparkes et al., 1991, Riemer et al., 2016). The sensitivity of AGP, although satisfactory and higher than that of all the other tests performed on blood, was lower than in previous reports (Giori et al., 2011). Nevertheless, the false negative cases had values consistent with inflammation (higher than the reference interval but lower than the cut-off consistent with FIP) which, in conjunction with other consistent laboratory results, may support the diagnosis of FIP. Interestingly, AGP showed also the lowest specificity and, although the LR- was not low enough to recommend the use of this test to rule out FIP, AGP was, along with the RT-nPCR 3'UTR, the most reliable test on blood indicating that values higher than the cut-off may support a clinical diagnosis in presence of other changes consistent with FIP (Paltrinieri et al., 2007). Based on the absolute specificity and on the relatively high LR-, also the molecular tests cannot be used to rule out FIP but they may support the diagnosis of FIP in the case of positive results.

Cytology and the RT-nPCR 3'UTR were the best test on effusion, despite the presence of false positive results. This latter event, however, was rare (only one case in our caseload, characterized also by a false positive cytological result) and it may be explained by the fact that non-specific inflammatory cytological patterns are found in many inflammatory conditions (Paltrinieri et al., 1999). Therefore, in accordance with previous reports, the cytology and the RT-nPCR 3'UTR cannot be used to confirm FIP (Giori et al., 2011, Kipar and Meli, 2014), but based on their high LR+ these are the tests of choice on effusion. The Δ TNC was specific but not as sensitive as expected, possibly due to the low cell concentration of the samples that provided false negative results also in study I. Hence, it may be used in addition to the other tests to support the diagnosis of FIP. The spike gene sequencing had a low sensitivity. Moreover, one false positive result was recorded, as

described by Porter and others (2014) who found the spike gene mutations described by Chang et al. (2012) in tissues of cats without FIP but in contrast with what reported by Longstaff et al. (2015), who found an absolute specificity of the spike gene sequencing on effusions. Therefore, the risk of false positive results, even if rare, make this test not optimal for the diagnosis of FIP. In tissues the sensitivity of RT-nPCR 3'UTR was high but not absolute, while the sensitivity of the spike gene was low. The negative results of the latter technique were due not only to the absence of the mutated nucleotides, but also to a negative result at the Spike PCR (data not shown) and to the resulting absence of sequencing templates. The RT-nPCR 3'UTR showed a low specificity, confirming again what already described about the spread of the virus in cats not affected by FIP and the resulting false positive results (Kipar et al., 2006b). On the other hand, the specificity of the S gene sequencing was high as on the other specimens. Thus, the risk of false positive results with the RT-nPCR 3'UTR is alarmingly high while the use of the S gene sequencing can be useful as a confirmatory test, but its use for the exclusion of FIP should be avoided based on the results of this study. A possible limitation of this study is that other mutations recently considered involved in the pathogenesis of FIP, such as the mutation of the 3c genes, have not been investigated. The inclusion of this additional molecular investigation would likely have provided more information on the pathogenesis of the disease and possibly on the inclusion of additional tests on the diagnostic workup. However, commercially available sequencing tests for FCoV are currently mostly focused on the detection of mutations in the S genes and therefore, being this a comparative study among tests routinely introduced in diagnostic panels, we preferred to focus our attention on tests that are already in use and well known in routine diagnostic procedures. In conclusion, this study confirmed that the diagnosis of FIP may be challenging in practice since none of the clinico-pathological or molecular test, alone, may diagnose FIP. However, the present study provided useful practical information on the possible combination of tests that increases the post-test probability of disease based on different clinical scenarios. Specifically, any test on blood, and especially the molecular tests, may support or confirm a clinical diagnosis of FIP. However, except for AGP, a negative result does not exclude the presence of this disease. When effusions are present, cytology should be preferred because it is rapid, cheap and easy to perform and may work either as confirmatory test (especially when the pre-test probability of disease is not high) or as an exclusion test in the case of negative results. The 3' UTR PCR has the same diagnostic performances than cytology but it is less practical to run. Conversely, the Δ TNC may confirm but

not exclude FIP and gene S sequencing on effusions cannot be recommended based on its lower performances in terms of LRs. On tissues, molecular tests may be run if immunohistochemistry is not available or if it provided dubious results. In this case, however, S gene sequencing should be preferred when histology is highly consistent with FIP while 3' UTR PCR when FIP is less likely. It would be interesting, in the future, to investigate the diagnostic performances of combined tests, which unfortunately has not been done in this study due to the low number of cases on which all the tests included in the study were performed.

V. Reverse transcriptase loop-mediated isothermal amplification for the detection of feline coronavirus

Material and methods

Caseload and RNA extraction

Thirty-two samples from 27 cats (11 feces, 9 blood, 8 effusions, and 4 mesenteric lymph nodes) submitted to the DIMEVET laboratory as part of a diagnostic panel for the clinical suspicion of FIP or, regarding feces, for screening purposes, were used. RNA extraction was performed using a NucleoSpin RNA Isolation Kit (Macherey-Nagel Bethlehem, PA). Whole blood and effusion samples were centrifuged (3,500 x *g*, 5 min) and the obtained pellets were suspended in 200 μ L of PBS by vigorous vortexing and stored at -20°C for further RNA extraction. Fecal samples were suspended at a final concentration of 10% (wt/vol) in PBS by vigorous vortexing. The supernatant was cleared by centrifugation (5,000 x *g*, 4 min) and stored at -20°C for further RNA extraction. For tissues, approximately 20 mg of sample were thinly shredded. All the further RNA extraction steps were performed according to the manufacturer's instruction.

RT-nPCR

The extracted RNA samples were tested for the presence of FCoV using a RT-nPCR targeting a 177 bp product of the highly conserved 3' UTR of the genome of both type I and type II FCoV (Herrewegh et al., 1995b). RT-nPCR positive FCoV RNA from a cat with FIP was used as positive control and RNase-free water as negative control. PCR products were visualized under UV transilluminator on a 2% agarose gel stained with ethidium bromide.

LAMP primers design and reaction set-up

The primers targeting the 3' UTR of the FCoVs were designed using the Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>) based on a 296 nucleotides sequence (28981 to 29277 bp) of the FCoV C1Je strain (accession number: DQ848678) (table 1). A Loopamp RNA amplification kit (RT-LAMP, New England Biolabs, UK) was used to perform RT-PCR LAMP and the reaction mixture was set up as follows: 1x Isothermal amplification buffer, 6 mM MgSO₄, 1.4 mM dNTPs, 320 U/mL of warm start DNA, 300 U/mL of warm start RTx Reverse Transcriptase, 2 μ M of both FIP and BIP primers, 0.2 μ M of both F3 and B3 primers, 1 μ M of both Loop F and Loop

B primers, 120 μ M of Hydroxynaphtol blue (HNB) dye (Sigma-Aldrich®) and 5 μ L of RNA template. The reaction mixture was then made up to 25 μ L with RNase-free water and incubated in a thermal cycler (MyCycler, Bio-Rad Laboratories, Hercules, CA, USA) for 1 hour at 63 °C followed by 10 minutes at 80 °C for heat inactivation. The same positive control used for traditional RT-nPCR, which tested positive also on the first RT-LAMP assay, was used as a positive control in the following RT-LAMP assays, , while RNase-free water was used as negative control.

PRIMER	GENOME TARGETING POSITION	SEQUENCE (5'-3')
F3	28982-29002	GCAACCCGATGTTTAAACTG
B3	29179-29162	CCATTGTTGGCTCGTCAT
FIP (F1c+F2)	F1c, 29065-29043; F2, 29003-29023	ACACGTGCTTACCATTCTGTACA- GTCTTTCCGAGGAATTACTGG
BIP (B1c+B2)	B1c, 29077-29101; B2, 29161-29141	CAAGCAACCCTATTGCATATTAGGA- AGCGGATCTTTAACTTCTCT
LOOP F	29042-29024	AGAGTAGACAGCGGATGA
LOOP B	29103-29128	GTTTAGATTTGATTTGGCAATGCTAG

Table 1. List of the primers (Primm Biotech, Italy) used in this study, based on the FCoV strain C1Je (GeneBank accession number: DQ848678).

The products of the reaction were then inspected both by eye, in order to detect the color turning from violet to sky blue in case of positive results with HNB (Goto et al., 2009), and under UV transilluminator on a 2% agarose gel stained with ethidium bromide in order to detect a ladder-like pattern in case of positive result (Parida et al., 2008) (Figure 1). Results obtained with RT-LAMP were then compared with those obtained with the RT-nPCR and the sensitivity and specificity of RT-LAMP obtained with both HNB and agarose gel were calculated.

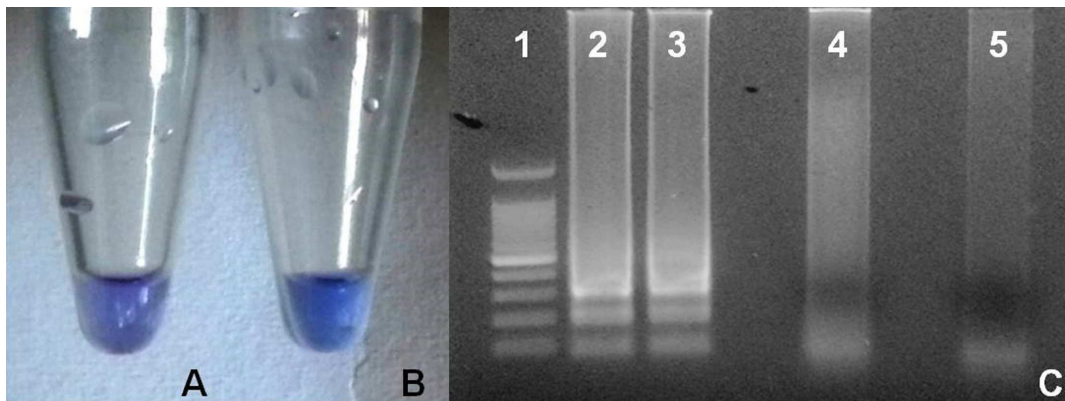


Figure 1. Evaluation methods of RT-LAMP for feline coronavirus. A. Negative result (violet) with hydroxynaphtol blue. B. Positive result (light blue) with hydroxynaphtol blue. C. Agarose gel

electrophoresis run showing 100 bp Ladder (lane 1); ladder like patterns typical of positive reaction (lanes 2 and 3); negative results (lanes 4 and 5).

Results and discussion

Results recorded in the different specimens are reported in table 1.

ID	Specimen	PCR	LAMP GEL	LAMP HNB
1	Feces	POS	POS	POS
2	Feces	POS	POS	POS
3	Feces	POS	POS	POS
4	Feces	POS	POS	NEG
5	Feces	POS	POS	NEG
6	Feces	POS	NEG	POS
7	Feces	POS	NEG	NEG
8	Feces	POS	NEG	NEG
9	Feces	POS	NEG	NEG
10	Feces	POS	NEG	NEG
11	Feces	NEG	NEG	NEG
Total positive results		10/11	5/11	4/11
12	Blood	POS	POS	POS
13	Blood	POS	NEG	POS
14	Blood	POS	NEG	NEG
15	Blood	POS	NEG	NEG
16	Blood	NEG	NEG	NEG
17	Blood	NEG	NEG	NEG
18	Blood	NEG	NEG	NEG
19	Blood	NEG	NEG	NEG
20	Blood	NEG	NEG	NEG
Total positive results		4/9	1/9	2/9
21	Effusion	POS	POS	POS
22	Effusion	POS	POS	POS
23	Effusion	POS	NEG	POS
24	Effusion	POS	NEG	NEG
25	Effusion	POS	NEG	NEG
26	Effusion	NEG	NEG	NEG
27	Effusion	NEG	NEG	NEG
28	Effusion	NEG	NEG	NEG
Total positive results		5/8	2/8	3/8
29	Tissue	POS	POS	POS
30	Tissue	POS	POS	POS
31	Tissue	POS	POS	POS
32	Tissue	NEG	NEG	NEG
Total positive results		3/4	3/4	3/4

Table 1. Results obtained on the samples tested with RT-nPCR (PCR) and LAMP and evaluated with agarose gel electrophoresis (LAMP GEL) and hydroxynaphtol blue dye (LAMP HNB).

All the negative samples using RT-nPCR were also negative by RT-LAMP, using both gel electrophoresis and HNB for the visualization of the results, leading to a 100% specificity. On the other hand, a conspicuous number of false negative results was recorded (11/22 for agarose gel and 10/22 for HNB) and the overall sensitivity was 50% and 54.5% using gel electrophoresis and HNB, respectively. The sensitivity of RT-LAMP was also different according to specimens: feces showed a sensitivity of 50% and 40%, with gel electrophoresis and HNB respectively. On blood, the sensitivity was 25% and 50%, with gel electrophoresis and HNB respectively while on effusions the sensitivity was 40% with both the visualization methods. Only on tissues, the sensitivity resulted to be absolute, but the number of tested samples was too low to be discussed in terms of diagnostic accuracy. Based on the results of this pilot study, RT-LAMP for FCoV, due to its high specificity, appears to be a solid molecular test to confirm the diagnosis of FCoV infection, and eventually to support a clinical diagnosis of FIP, when performed on specimens from cats with an high pre-test probability of FIP (e.g. cats with clinical signs and laboratory findings consistent with FIP, like effusions with physico-chemical and cytological features consistent with FIP) but its low sensitivity makes this test not reliable in case of negative results (Pedersen, 2009). In the case the sensitivity of the test might be ameliorated through further studies, the RT-LAMP could be extremely useful, due to its low costs and rapidity, in those situations where the detection of FCoV must be repeated over time and on a high number of cats (e.g. breeding catteries). An additional future perspective would be the optimization of the test to obtain quantitative results, possibly by establishing a standard curve of color intensity using RNA samples with known viral load (e.g. quantification of RNA copies by quantitative PCR techniques).

VI. Feline coronavirus spike protein in cats with and without feline infectious peritonitis

Material and methods

A large biobank consisting of variable clinical specimens (tissues; body cavity fluid; feces) obtained from cats deceased for both FIP and other diseases (e.g. neoplasia, cardiac failure) and stored at -80°C at School of Veterinary Science (University of Bristol, UK) was used. For every cat from which samples were stored in the biobank, a full clinical evaluation along with histology and immunohistochemistry for the detection of feline coronavirus were available.

Collection of samples

Cats euthanized due to suspected FIP or to other diseases at the Small Animal Hospital (Langford Veterinary Services, University of Bristol) underwent necropsy and sample collection within two hours of death. In particular, at least five samples of 0.125 cm^3 (maximum 5 mm in width/length/depth) from each of colon, small distal intestine, mesenteric lymph node, spleen, liver, kidneys, omentum and lungs were sectioned and immediately placed in RNA preservative *RNAlater*[®] (Life Technologies). Other kind of tissues (e.g. brain, eyes, pancreas) were also occasionally collected based on gross findings. A sample of feces and, if present, body cavity effusions (abdominal, thoracic, pericardial, CSF) were also collected in plain tubes. Concurrently, tissue samples from the organs as reported above were collected into 10% neutral-buffered formalin for histopathology and immunohistochemistry tests. Samples collected in *RNAlater* were refrigerated for 24-48 hours, prior to storage at -80°C . Fecal samples were immediately stored at -80°C .

RNA extraction and qPCR

Regarding tissues, RNA was purified from 20 mg tissue using a NucleoSpin RNA II Kit (Macherey-Nagel) according to manufacturer's instructions, following disruption using a Qiagen Retsch MM300 TissueLyser (Qiagen, Hilden, Germany). Regarding feces, RNA was purified from 10 mg feces (100 μl of 500 mg of feces suspended in 4,5 mL of phosphate buffered saline (PBS), or fecal swabs (resuspended in 100 μL PBS), using the Chemagic 360 instrument (Perkin-Elmer) in combination with Chemagic body fluids NA kit (Perkin-Elmer). Regarding fluids, RNA was purified

from 100 µl fluid using the Chemagic 360 instrument in combination with Chemagic body fluids NA kit. The resulting RNA was stored at -80°C until further analysis.

All samples underwent reverse transcription (RT) on a MJ Mini Gradient Thermal Cycler using the ImProm II Reverse Transcriptase (Promega) kit. The reaction mixture comprised 4 µL of 5X ImProm II buffer, 1 µL of random hexamers (0.5 µg/ µL), 2.4 µL of 25 mM MgCl₂, 1 µL of dNTPs (10 mM each), 1 µL of ImProm RT enzyme, 10 µL of RNA template and was made up to 20 µL with RNase-free water. Thermal conditions were: 20 °C for 5 minutes, 42 °C for 30 minutes, 70 °C for 15 minutes and 10 °C hold. The obtained complementary DNA was added to 30 µL of RNase-free water and stored at -20 °C for further analyses or immediately used. For fecal RT samples, an RNA internal amplification control was used to check the presence of RT inhibitors.

A quantitative PCR (qPCR) for the detection of FCoV was performed on all the cDNA samples. An Agilent Mx3005P qPCR System (Agilent Technologies) thermocycler was used. The reaction mixture was prepared as follows: 12.5 µL of 2x GoTaq polymerase, 0.5 µL each of 10 µM forward (P009:AGCAACTACTGCCACRGGAT, position 26655-26674 of the FCoV strain 79-11461) and reverse (P010:GGAAGGTTTCATCTCCCCAGT, position 26826-26807 of the FCoV strain 79-11461) primers, 0.125 µL of 5 µM FAM *TaqMan* probe, 1.25 µL of 50 nM MgCl₂, 5 µL of cDNA template, and made up to 25 µL with RNase-free water. The primers, targeting a conserved 171 bp region between the M and the N gene, were previously described by Dye et al. (2008). The following thermal profile was used: 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds, 55 °C for 15 seconds and 72 °C for 15 seconds. Feline coronavirus cDNA was used as a positive control and RNase-free water as a negative control. Reactions that did not reach the threshold cycle (Ct) value after 40 cycles were categorized as negative and a relative copy number of 1 was assigned to a Ct value of 40.

Pyrosequencing

All samples that were positive by FCoV qRT-PCR underwent conventional PCR to amplify a 153 bp DNA fragment, which including the sequences encoding for positions 1058 and 1060 of the spike protein. The PCR was performed on a MJ Mini Gradient Thermal Cycle with a reaction mixture containing: 12.5 µL of 2x GoTaq polymerase, 1 µL each of 10 µM forward (F614: GCHCARTATTAYAATGGCATAATGG, position 23436-23460 of the FCoV C1Je2 strain) and biotinylated reverse (R766: BIO-AAGYCTRGCYTGYACTTGCAT, position 23588-23568 of the FCoV C1Je2 strain) primers, 2 µL of the template cDNA, and made up to 25 µL with RNase-free water.

The following thermal profile was used: 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 15 seconds, 52 °C for 20 seconds and 72 °C for 20 seconds. The amplification products were stored at -20 °C for further analyses or immediately pyrosequencing. Samples that did not pyrosequence were amplified for 50 cycles.

The pyrosequencing used in this study is aimed to detect the two mutations described by Chang et al (2012). At first, the PCR biotinylated products underwent the chemical separation in single strands through the binding with streptavidin Sepharose beads (Fisher). For each sample, the binding reaction mixture was prepared as follows: 40 µL of PyroMark binding buffer (Qiagen), 1 µL of streptavidin Sepharose beads and 25 µL of PCR product, made up to 80 µL total with RNase free water. The mixture was placed on a shaking block for 10 min at 1400 rpm. To prepare the sequencing plate, the following reaction mixture was prepared for each sample: 38 µL of PyroMark annealing buffer (Qiagen) and 2 µL of 10 µM sequencing primer (S680: ACAGCCTCDTTAATAGGVGGATG, position 23502-23524 of the FCoV C1Je2 strain). Forty µL of the sequencing mix were added to the apposite wells of the sequencing plates and the plate was placed on the pyrosequencing workstation of the PyroMark Q96 (Qiagen).

The trays of the pyrosequencing workstation were filled with washing solution buffer (10 mM Tris-OAc pH 7.6), denaturing solution (0.2 M NaOH), 70% ethanol and distilled water. The streptavidin beads already bound with the S gene qPCR biotinylated products were then gently aspirated by the pyrosequencing bead collector connected to a vacuum, after a cleaning step of the bead collector with distilled H₂O. The bead collector was then placed in the 70% ethanol for 5 seconds, in the denaturing solution for 5 seconds and in the wash solution for 10 seconds. The vacuum was then turned off and the bead collector was placed in the pyrosequencing plate and gently agitated for 40 seconds to let the beads to detach. To let the primers to anneal to the the beads, the plate was heated at 80 °C for 2 minutes on a plate holder, and then left to cool for 5 minutes in the PyroMark Q96. In the meanwhile, the pyrosequencing cartridge was set with the following reagents in the wells and with the volumes indicated by the PyroMark Q96 software: PyroMark Gold Q96 enzyme, substrate and dNTPs (Qiagen). The dispensation order of the dNTPs was: CGCTCATG. The cartridge was then placed into the PyroMark Q96 and the assay was run.

After the first experiments, the pyrosequencing of the target sequence was doubled, in order to look for the two possible mutations in two separated work sessions. This decision was taken after the failing of some pyrosequencing, possibly due to the formation of loops in the chosen primer or to the variability of the sequence. The second pyrosequencing (aimed to detect the mutation

S1060A) was performed on each sample after the first above described and with the same protocol, except for the use of a different sequencing primer (S693 instead of S680) and dispensation order of the dNTPs.

Results analysis

Cats were classified as FIP, non-FIP, or inconclusive based on histology and immunohistochemistry results. Cats were assigned to the FIP group if histology revealed the presence of the typical histological lesions (e.g. pyogranulomas-granulomas, vasculitis, fibrinoid necrosis) and if the FCoV antigen was found within the lesion macrophages.

All the pyrograms have been downloaded from the PyroMark Q96 software and are currently under evaluation. Each pyrogram will be analyzed in order to assign the corresponding codon to the peaks. To every sample obtained from each cat, the Ct values and the presence/absence of one or both the mutations described by Chang et al (2012) will be assigned.

Therefore, results of this part of study VI are too preliminary to provide useful information on the pathogenesis of FIP/FCoV infection and are not included in the current version of the thesis.

VI.1. Feline coronavirus shedding in cats with and without feline infectious peritonitis.

Briefly, 83 samples of feces and seven fecal swabs were collected from 90 cats for which histopathology results were also available. Cats were classified as FIP, non-FIP, or inconclusive based on histology and immunohistochemistry results. Cats were assigned to the FIP group if histology revealed the presence of the typical histological lesions (e.g. pyogranulomas-granulomas, vasculitis, fibrinoid necrosis) and if the FCoV antigen was found within the lesion macrophages. Inconclusive cats (histology consistent with FIP but with negative results at immunohistochemistry for FCoV) were not included in the study).

Results and discussion

Forty-eight cats were classified as FIP and 42 as non-FIP.

Thirtyone (31/48) samples from the FIP group were FCoV RT-qPCR positive as well as 12 cats (12/42) from the non-FIP group. Pyrograms were unreadable for five FIP cats and for two non-FIP cats. The failure in the pyrosequencing could be attributed to a failure of the sequencing primers more than to a failure of the PCR targeting the S gene, this latter finding being confirmed with the specific band appearance when the PCR products were run on the agarose gel. Another explanation could be the formation of loop/helix structures in the sequencing primers, as predicted using M-Fold web server which also recorded melting temperature higher than those used in the pyrosequencing. The low copy numbers produced with the RT-qPCR could be another explanation as well the presence of type II FCoVs in the feces. The spike gene protein of this serotype, in fact, has sequences more similar to that of the CCoV and could prevent the binding of the primers.

The remaining pyrograms were from 26 cats with FIP and from 10 without FIP. Thirteen FIP cats (13/26) were shedding the FCoV with the M1058L mutation described by Chang et al. (2012) as well as two (2/10) of the non-FIP cats.

The results obtained are in contrast with those reported in previous papers, where the fecal samples obtained from non FIP cats did not show the M1058L mutation (Chang et al., 2012; Porter et al., 2014). On the other hand, this study demonstrates that FIP cats are more likely to shed FCoV with feces, at least twice as non-FIP cats. Moreover, it is possible that the spread of mutated FCoVs may play a role in the pathogenesis of FIP, differently from what reported in previous studies (Pedersen, 2009). This latter finding could have not been investigated in this study, but it

would be interesting to determine the presence of 3c mutations in the same fecal samples, since its truncations seems to increase the viral pathogenicity (Hora et al., 2016). Another approach could be to evaluate the infectivity of these strains for enterocyte cell lines, as tried in recent studies (Desmarets et al., 2013).

CONCLUSIONS

This thesis was aimed to find new approaches for the diagnosis of FIP and FCoV infection, since one of the main problematic aspects of this disease is the absence of a gold standard for the diagnosis *in vivo*. A new LAMP test was developed and, although further studies are needed to completely investigate the diagnostic potential of this test, its use in clinical practice could help to add more information during the diagnostic as well as the screening procedure, due to its advantageous properties. Within this thesis The diagnostic potential of the Δ TNC has been fully exploited through two sequential studies and this test may become extensively available in routine practice, either on fresh or on frozen effusions. On the other hand, one of the classic test to perform on serum (i.e. SPE) was found to be less reliable for the diagnosis of FIP, showing the need for the development of additional tests and for deepening the reason of this change. The most useful tests to perform *in vivo* were determined, including a molecular test recently proposed to discriminate the two FCoV pathotypes. This latter test was also investigated in a wide number of samples during the externship at the University of Bristol and, although most of the results collected during the externship are very preliminary and ultimately did not allow us to provide additional information on the pathogenesis of FIP, these results may be of interest to further investigate the genomic variability through the completion of the analysis so far performed, or through similar future studies on this topic. Nonetheless, the molecular study run during the externship showed for the first time the presence of the mutated virus also in feces of non FIP cat. This result ultimately open some interesting questions on the pathogenesis of the disease and on the need of novel testing strategies to control the spread of infection in multicat households.

Specifically, the main conclusions of this thesis are:

- ❖ The Δ TNC measurement on feline effusions has a high diagnostic accuracy for the diagnosis of FIP. Two cut-offs were determined. A Δ TNC >1.7 FIP is suggestive of FIP, while a Δ TNC >3.4 can be considered diagnostic for FIP.
- ❖ The Δ TNC measurement can be performed also on effusion supernatants (e.g. on frozen, stored sample) after the addition of feline blood samples in 1:10 dilution.
- ❖ Serum protein electrophoresis performed in FIP cats in recent years showed a decrease in hyperproteinemia and hypergammaglobulinemia, compared with both previous years and

with reference intervals. This finding suggests possible changes in the virus-host interaction.

- ❖ The comparison between different tests for the diagnosis of FIP identified AGP on blood and cytology and RT-nPCR on effusions as the best tests to perform in vivo. On tissues, RT-PCR and spike gene sequencing may be useful to exclude and confirm the disease, respectively.
- ❖ With the settings used in this study, RT-LAMP showed absolute specificity but poor sensitivity in all the samples except on the few number of tissues evaluated. Thus, it could be used as a confirmatory test but not to exclude FCoV infections.
- ❖ FIP cats are more prone to shed FCoV in feces. Moreover, the M1058L was found in feces of cats died for FIP but also for diseases other than FIP. The shedding of mutated FCoV may have a role in the pathogenesis of FIP.

Publications

International peer reviewed papers

- Giordano, A., Stranieri, A., Rossi, G., Paltrinieri, S., 2015. High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis. *Veterinary Clinical Pathology* 44: 295-302.
- Stranieri, A., Ferrari, R., Zanzani, S., Rossi, G. 2016. Sysmex XT-2000iV scattergram analysis in a cat with basophilia. *Veterinary Clinical Pathology* 45(2):225-228.
- Stranieri, A., Giordano, A., Bo, S., Braghiroli, C., Paltrinieri, S. 2016. Frequency of electrophoretic changes consistent with feline infectious peritonitis in two different time periods (2004-2009 vs 2013-2014). *Journal of Feline Medicine and Surgery*. Epub ahead of print, doi: 1098612X16664389
- Stranieri, A., Paltrinieri, S., Giordano, A. 2016. A reliable method to diagnose feline infectious peritonitis (FIP) on frozen supernatants from cavitory effusions. *Journal of Veterinary Diagnostic Investigation*, E-pub ahead of print. doi:10.1177/1040638716689115
- Stranieri, A., Giordano, A., Paltrinieri, S., Giudice, C., Cannito, V., Lauzi, S., 2016 Comparison of diagnostic accuracy for feline infectious peritonitis of various laboratory tests performed on different specimens. *Veterinary Record* [Under review]
- Stranieri A., Lauzi, S., Giordano, A., Paltrinieri, S., 2016. Reverse transcriptase loop-mediated isothermal amplification for the detection of feline coronavirus. *Journal of Virological Methods*, 243: 105-108. [E-pub ahead of print]

Abstracts

- Ibba F., Stranieri A., Xenoulis V., Paltrinieri S., 2015. Urinary γ -glutamyltransferase (GGT) as a marker of tubular damage in dogs with leishmaniasis, using sodium dodecylsulphate (SDS) electrophoresis as a reference method. 16th European Society of Veterinary Clinical Pathology (ESVCP) Annual Meeting, 1-4th October 2014, Milan, Italy . Published in: *Veterinary Clinical Pathology*, 44(1): E1-E23.
- Stranieri, A., Paltrinieri, S., Giordano, A., 2014. A modified method to evaluate "Delta-WBC" using Sysmex XT-2000iv in supernatants of centrifuged feline effusions. 49th American Society of Veterinary Clinical Pathology (ASVCP) annual meeting, 8-12th November 2014, Atlanta, Georgia, USA. Published in: *Veterinary Clinical Pathology*, 43: E8-E21.
- Stranieri A., Lauzi, S., Giudice, C., Cannito, V., Giordano, A., Paltrinieri S. (2015) Comparison between the diagnostic accuracy of clinico-pathological and molecular tests for feline infectious peritonitis (FIP). 25th European College of Veterinary Internal

Medicine-Companion Animals (ECVIM-CA) annual meeting, 10-12th September 2015, Lisbon, Portugal. Published in: Journal of Veterinary Internal Medicine, 30(1): 348–439.

- Stranieri A., Lauzi, S., Giordano, A., Paltrinieri, S. A Reverse Transcription Loop-Mediated Isothermal Amplification (RT-Lamp) Assay for Feline Coronavirus. ISCAID Symposium, 16-19 October 2016, Bristol, UK
- Barker, E.N., Stranieri, A., Helps, C., Porter, E.L., Davidson, A.D., Day, M.J., Kipar, A., Tasker, S. Feline Coronavirus Shedding in Cats With and Without Feline Infectious Peritonitis. ISCAID Symposium, 16-19 October 2016, Bristol, UK

Oral presentations at international meetings

- Stranieri, A., Ferrari, R., Zanzani, S., Rossi, G. (2014) "A peculiar Sysmex scattergram in a cat affected by feline injection site sarcoma" 16th ESVCP Annual Meeting. October 1st-4th, Milan, Italy
- Stranieri A., Lauzi, S., Giudice, C., Cannito, V., Giordano, A., Paltrinieri S. (2015) Comparison between the diagnostic accuracy of clinico-pathological and molecular tests for feline infectious peritonitis (FIP). 25th ECVIM-CA Congress. September 10th-12th, Lisbon, Portugal

Co-supervision of Degree thesis

- Valentina Cannito: Peritonite infettiva felina (FIP): confronto tra test diagnostici e potenzialità del gene Spike (S) del coronavirus in studi filogenetici. Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Angelica Stranieri. December 2014
- Chiara Braghiroli: Patterns elettroforetici in gatti con fip in due differenti archi temporali (2004-2009 e 2012-2014): variazione del potenziale diagnostico. Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Angelica Stranieri. October, 2015
- Daniela Cortiana: Basofilia nel gatto: prevalenza, associazione con stati patologici e possibili approcci strumentali. Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Angelica Stranieri. October, 2015
- Granauro Silvia: Elettroforesi siero proteica e malattie parassitarie: principali patterns elettroforetici in gatti sieropositivi e malati. Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Angelica Stranieri. Maggio 2016

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