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HIGH DIAGNOSTIC ACCURACY OF THE SYSMEX XT-2000iV DELTA TOTAL NUCLEATED CELLS (ΔTNC) ON EFFUSIONS FOR FELINE INFECTIOUS PERITONITIS

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HIGH DIAGNOSTIC ACCURACY OF THE SYSMEX XT-2000iV DELTA TOTAL NUCLEATED CELLS (ΔTNC) ON EFFUSIONS FOR FELINE INFECTIOUS PERITONITIS

Running Head: Accuracy of Sysmex counts on FIP effusions

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Abstract

Background: The ΔWBC (the ratio between DIFF and BASO counts of the Sysmex XT-2000iV), hereafter defined as ΔTNC (total nucleated cells), is high in effusions due to feline infectious peritonitis (FIP), since cells are entrapped in fibrin clots formed in the BASO reagent. Similar clots form in the Rivalta's test, that has a high diagnostic accuracy for FIP. *Objectives*: to determine the diagnostic accuracy for FIP and the ideal cutoff of the ΔTNC. *Methods*. After a retrospective search of our database, DIFF and BASO counts and the ΔTNC from cats with and without FIP were compared to each other. Sensitivity, specificity, ~~and~~ positive and negative likelihood ratios (LR+, LR-) were calculated. A ROC curve was designed to determine the cutoff. *Results*: Effusions from 2~~0~~5 FIP and 31 non-FIP cats were analyzed. The ΔTNC was significantly higher (P<0.001) and BASO and DIFF counts were significantly lower (P<0.001 and P<0.05) in FIP (median values: 9.3; 0.2; 1.5~~12.5±11.2; 0.5±1.1; 4.5±7.4~~) than in non-FIP cats (1.0; 10.1; 9.1~~1.1±0.4; 43.5±127.0; 52.6±164.6~~). Only two FIP cats with atypical effusions ~~(a transudate-like and a pericardial effusion)~~ had a ΔTNC <3.0. The cutoff identified by the ROC curve (area under curve: 0.9~~5~~; P<0.001) was 1.7 (Sens=90.0~~2~~%; Spec=90.3~~3.5~~%; LR+=~~14.3~~13.9; LR-=0.1). A ΔTNC >2.5 has 100% specificity. *Conclusions*: the ΔTNC has a high diagnostic accuracy for FIP and provides ~~both~~ an estimate of precipitable proteins, as the Rivalta's test, and information about cell counts. However, fibrin clots lowers the BASO counts. Therefore, when FIP is suspected, the ΔTNC is preferable to the ~~default~~ WBC count generated by the BASO channel.

Keywords: Cat; Coronavirus; Likelihood ratio; Rivalta's test; Sensitivity; Specificity

Introduction

Feline Infectious Peritonitis (FIP) is a ubiquitous, lethal disease caused by the Feline coronavirus (FCoV) and triggered by an excessive immune response of cats infected with mutated FCoVs variants.[1]

The ante-mortem diagnosis of FIP is always challenging, especially in its non effusive ('dry') form, due to the variable clinical signs and the poor specificity of many laboratory assays. Among these, serum proteins electrophoresis and the α1-acid glycoprotein (AGP) measurement may support a clinical suspicion of FIP.[2-4,6] ~~In cats affected by FIP, serum proteins electrophoresis shows hypoalbuminemia and hyperglobulinemia with an increase of α_2- and γ-globulins,[5] AGP is an acute phase protein that increases during inflammatory and infectious disease and can reach very high levels (>1.5 mg/mL) in cats affected with FIP.[6]~~ Also these tests, however, cannot provide a confirmatory diagnosis of FIP. [2] On the other hand, the effusive ('wet') form is easier to diagnose, based on the signalment and history ~~(e.g. young age; persistent fever; weight loss; ascites)~~, on the results of the biochemical tests mentioned above and especially on the analysis of effusions.

Macroscopically, the FIP effusion is yellow, turbid, sticky and it often contains fibrin strands. The protein content is usually high ~~(more than 3.5 g/dL)~~ with a decreased albumin to globulin ratio.[7] Cell count ranges from 2 to 6 x 10^{9}/~~µ~~L, sometimes even to 30 x 10^{9}/~~µ~~L,[8] and the cytological examination, which is only highly suggestive but not definitely diagnostic for FIP, shows mostly non-degenerated neutrophils, macrophages, lymphocytes and rare plasma cells on a ~~A typical~~ proteinaceous background ~~is almost always seen~~.[1,9] ~~Unfortunately, even cytology of the effusions, although highly suggestive for FIP, is not completely diagnostic.[10] The detection of FCoVs within macrophages in the effusion by a direct immunofluorescence was considered highly specific[11] but poorly sensitive,[2] but recently also the specificity of this test has been questioned.[12]~~

Conversely, the Rivalta's test has been recently proposed as one test with high accuracy for the diagnosis of FIP.[10,13]

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The Rivalta's test is an inexpensive, easy to perform assay, used to differentiate transudates from exudates. ~~The principle of the test is very simple and it is based on the addition of a drop of effusion into an acidic solution: if the solution remains clear, the test is negative. If the drop retains its shape, flows to the bottom of the tube or adheres to the surface, the test is positive.[2]~~

The positive reaction to the acetic acid ~~is~~ induced by the presence of a high concentration of proteins, fibrinogen and other acute phase proteins, which clots into the tube.[1,14] In turn, these compounds are particularly abundant in effusions from cats with FIP but can ~~be~~ also be present in effusions due to ~~pathological conditions other than FIP, such as~~ bacterial peritonitis and pleuritis or lymphoma.

~~In these cases, however, a culture or a cytological examination of the exudate can help to differentiate bacterial infection or tumors from FIP.[8]~~

Therefore, in feline medicine the Rivalta's test, coupled with cytology of the effusion, may be a quick way to distinguish FIP effusions from other type of effusions. Several studies demonstrate the diagnostic utility of Rivalta's test for FIP because of its high sensitivity- and accuracy.[10] ~~and its good positive (PPV) and negative predictive value (NPV).[13]~~

In a recent study on canine and feline effusions it has been shown that the Delta (Δ) TNC (the ratio between total nucleated cell counts – TNCC – in the DIFF and BASO channel of the laser counter Sysmex XT-2000iV, reported by the instrument as "ΔWBC"), is higher in effusions of cats affected by FIP than in other effusions.[1,5] The BASO channel uses an acidic reagent that induces, except for basophils, the collapse of the cells. In FIP effusions, this reagent induces also the formation of a clot that entraps the cells and lead to a low BASO count. Therefore this mechanism, responsible for the increase of the ΔTNC, is very similar to the analytical principle of the Rivalta's test.

The aim of this study is to determine, according to the STARD (Standards for Reporting of Diagnostic Accuracy) approach,[1,6,14,7] the diagnostic accuracy of the ΔTNC for FIP on a larger number of cases and to assess whether it may have the same diagnostic utility than that reported for

the Rivalta's test[103] and to define the ~~best~~ cutoff value of ΔTNC that minimize false positive and negative results for the diagnosis of FIP.

Material and methods

Re~~s~~trospective selection of cases

This was a retrospective study performed on data from effusion samples submitted to our Institution within our routine diagnostic activity and collected ~~for diagnostic purposes~~ under informed content of the owners. Therefore, in accordance with the guidelines of our Institution, a formal approval from the Ethical Committee was not required.

The database of our Institution regarding the period June 2009 – June 2013 was searched to select feline intracavitary effusions that had been analyzed with the Sysmex laser counter as described below.

Data were then examined to select cases to be included in this study based on the following inclusion and exclusion criteria:

The inclusion criteria were:

- Presence of complete information about physico-chemical analysis of the effusion (i.e. specific gravity and protein content estimated by refractometric analysis)
- Presence of exhaustive information about the final diagnosis according to the criteria described below
- Availability in the archive of our Institution of cyto-centrifuged slides to assess the cytological pattern of effusions in those cases on which no information on cytology were reported in the database

The exclusion criteria were:

- Absence of information regarding the follow up
- Absence of information on cytological features of effusions

- Absence of slides to verify the cytological pattern in those cases on which no information on cytology was reported in the database.
- Unclear or non-conclusive cytological findings in those cases on which no information were available in the database but slides were stored in the archive

Based on these criteria, cats were considered as affected by FIP when results of serum protein electrophoresis (and/or of the effusion), measurement of the serum concentration of AGP and cytology of the effusions were consistent with FIP and the disease was confirmed post-mortem by necropsy, histology and positive immunohistochemistry for FCoVs performed as described in a previous study[4] ~~or if the follow up revealed a progressive worsening of the clinical condition in spite of antibiotic or other supportive therapies and the persistency of laboratory changes consistent with FIP~~. Conversely, cats were considered as not affected by FIP if cytology or bacteriology of the effusion diagnosed a disease other than FIP, eventually confirmed by necropsy and histology, or if the follow up revealed a rapid improvement of the clinical conditions after treatments, as better specified in the results section.

All the samples were submitted to our laboratory for routine diagnostic purposes and were subjected to cell counts, ~~physico-chemical analysis of the fluid (evaluation of the specific gravity and refractometric estimation of the protein content~~measurement of specific gravity and protein concentration by refractometry (Clinical refractometer Mod. 105 Sper Scientific, Scottsdale, USA~~)~~ and ~~by~~ cytological analysis. ~~When possible, n~~Necropsies~~y~~ and additional post-mortem tests were performed at the routine necropsy service of our Department.

In all the cases above, cytology and results of biochemical tests have been evaluated by two ECVCP certified clinical pathologists that were unaware of the results of the Sysmex counts.

Analytical method

According to the SOP's of our laboratory only~~All of th~~e effusions, collected in EDTA tubes, and submitted to the lab no more than 12-18 hours after sampling have been analyzed ~~within 12 hours~~

~~from sampling~~ on the Sysmex XT-2000iV (Sysmex Europe GmbH, Norderstedt, Germany) analyzer to determine the total nucleated cell count (TNCC) provided by both the DIFF (TNCC-DIFF) and BASO (TNCC-BASO) channels, as well as the ΔTNC. Specifically, the DIFF channel classifies cells based on complexity and nucleic acid content. The BASO channel classifies cells based on volume and the complexity of cellular residues produced after contact with an acidic reagent that, in people, collapses all the nucleated cells except basophils.[1~~5~~8] Since effusions include cells other than WBCs, the total WBC counts and the ΔWBC generated by the instrument, have been defined as TNCC and ΔTNC for the purpose of this study.[1~~2~~5]

Evaluation of diagnostic sensitivity and specificity

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

Results regarding the TNCC-DIFF, the TNCC-BASO and the ΔTNC recorded in cats with and without FIP have been compared to each other with a non-parametric *t*-test (Mann-Withney *U* test), using the 95% confidence interval (CI) as a measure of uncertainty.

In order to assess the diagnostic accuracy of the ΔTNC, the number of true positive (TP), false positive (FP) true negative (TN) false negative (FN) results has been calculated as follows:

TP = samples from cats with FIP with a ΔTNC higher than each operating point ~~value~~

TN = samples from cats without FIP with a ΔTNC lower than each operating point ~~value~~

FP = samples from cats without FIP with a ΔTNC higher than each operating point ~~value~~

FN = samples from cats with FIP with a ΔTNC lower than each operating point ~~value~~

Using these numbers, sensitivity and specificity were calculated using standard formulae[1~~6~~9] ~~-~~and using the 95% confidence interval (CI) as a measure of uncertainty. In addition, the positive and negative likelihood ratio (LR+ and LR-) ~~was~~ were calculated using the formula~~e~~: LR+ = (sens)/(1-spec) and LR- = (1-spec)/(sens).[2~~1~~7~~0~~]

Finally, Receiver Operating Characteristic curves (ROC curves) were designed by plotting Sens vs. 1-spec, in order to determine the discriminating power of the ΔTNC to identify cats with FIP.[2170] In addition, the optimal cut-off value, corresponding to the operating point ~~value~~ closer to the upper left corner of the graph was identified.

Analytical precision and accuracy

Analytical precision and accuracy of Sysmex counts on feline effusions not associated with FIP were already evaluated in the previous study.[125] Specifically, intra assay coefficient of variation (CVs) accounted for 11.5% for TNCC-DIFF and 0.5% for TNCC-BASO and regression coefficients of samples read after serial dilutions were higher than 0.99 for both TNCC-DIFF and TNCC-BASO. In the same study a poor repeatability and linearity under dilution of a few samples from cats with FIP were reported but no information on the actual repeatability and linearity under dilution of Sysmex readings of TNCC-DIFF and TNCC-BASO of effusions from cats with FIP, nor information about precision and accuracy of the ΔTNC were reported.

Therefore, in the current study repeatability has been assessed only on two FIP samples with a high ΔTNC and on two samples with a normal ΔTNC by analyzing the samples 5 consecutive times in 1 day and by calculating the CVs with the formula: CV = mean/SD x 100. To assess linearity under dilution, one sample with high ΔTNC and one with normal ΔTNC were serially diluted 1:1, 1:3, 1:7, and 1:15 (vol/vol) with isotonic saline, leading to dilutions corresponding to 50%, 25%, 12.5% and 6.25% of the undiluted fluid, respectively. Samples have been then analyzed on the Sysmex as described above. Linearity has been determined by comparing by linear regression analysis the expected values for each dilution to the values released by the instrument.~~.~~

Results

Results of the retrospective search and distribution of cases per group

The retrospective search of the database identified 67 feline effusions coming from cats of different age, sex and breed processed during the study period (June 2009-June 2013) (Figure 1). Among these, 16~~1~~ have been excluded due to non-conclusive cytological findings and to the lack of information on the follow up or on post-mortem tests

The remaining 516 effusions have been grouped as follows:

Group A: FIP (n=~~25~~20): In all these cases, except 2, the physico-chemical features and cytology of the effusions were consistent with FIP, showing usually non-degenerated neutrophils, macrophages, lymphocytes and rare plasma cells and mesothelial cells along a granular proteinaceous background.

The two cases of FIP with "atypical" findings in the effusion were the following: in both cats blood findings were consistent with FIP (polyclonal gammopathy and very high AGP concentration) but the effusion of cat #5 ~~had an abdominal fluid with~~had low protein content (17 g/L), low specific gravity (1,010) and low cellularity (0.13 x 10^{9}~~3~~/~~µ~~L), with ~~and low specific gravity (1,010). Serum protein electrophoresis of this cat revealed the typical polyclonal gammopathy and a very severe hypoalbuminemia (13 g/L, ref. interval 23-37 g/L), associated with an extremely high serum concentration of AGP (9 mg/mLl; ref interval: 0.34-0.56 mg/mL). Cytology evidenced r~~rare neutrophils and mesothelial cells in the absence of the ~~typical~~ proteinaceous background typical of FIP effusion and the ~~. Necropsy evidenced a fibrinous serositis typical of FIP in all the abdominal organs. However, these lesions were associated with multiple hemorrhages (figure 2A).~~pericardial effusion of ~~C~~cat #25 ~~had a pericardial effusion on which the proteinaceous background typical of FIP effusions was not clearly evident, and cytology~~ revealed a high number of ~~large round cells likely interpretable as~~ reactive mesothelial cells, sometimes with evident cytophagia and a weak proteinaceous background~~, along with a moderate number of non-degenerated neutrophils and lymphocytes (figure 2C). Also in this case serum protein electrophoresis was consistent with FIP and the AGP concentration was severely increased (3.7 mg/mL). In a few days the cat developed~~

~~also a pleural effusion and was euthanized.~~ . However, in both cases n~~N~~ecropsy revealed ~~the simultaneous presence of fibrinous pericarditis and pleuritis associated with~~ the typical subserosal fibrinous lesions (associated with multiple hemorrhages in cat #5) and the diagnosis of FIP was confirmed by ~~. In both cases,~~ histology ~~confirmed the presence of fibrinous serositis~~ and by the immunohistochemical detection of ~~stry evidenced~~ intralesional FCoVs ~~(figure 2B, 2D)~~.

Necropsy, histology and immunohistochemistry confirmed FIP on additional 18 cats. ~~Therefore, the total number of cats on which FIP was confirmed post mortem accounted for 20 cats. In the remaining 5 cats with clinical, cytological and physico-chemical findings consistent with FIP, the diagnosis was further supported by the presence of increased α_2- and γ-globulin in electrophoretograms of serum and effusions and by a serum concentration of AGP higher than 1.5 mg/mL mg/mL, that is considered a threshold potentially useful to differentiate cats with FIP from cats with other diseases.[3,6] Specifically, the AGP concentration in these cats ranged from 1.9 to 5.4 mg/mL (mean ± SD: 3.4 ± 1.4 mg/mL; median: 3.2 mg/mL). Furthermore, these 5 cats died in a few weeks due to a progressive worsening of the clinical conditions in spite of supportive and antibiotic treatments, and in 3 cases clinico-pathological tests on serum and effusions repeated during the follow up were still consistent with FIP.~~

Group B: non FIP (31 cats): this group included neoplastic effusions (n=20) due to lymphoma (n=10) or epithelial tumors (n=8), diagnosed by cytology of the effusion, thymoma (n=1) and hemangiosarcoma (n=1) diagnosed by the detection of unclassified atypical cells in the effusion and by diagnostic imaging, followed, in the case of the hemangiosarcoma, by necroscopic and histologic findings; exudates associated with inflammatory conditions (n=5) diagnosed by cytology of the effusion, that revealed a prevalent population of neutrophils, in 3 cases associated with positive bacteriology on the effusion and in 2 cases associated with clinical and laboratory findings consistent with feline cholangiohepatitis. All these cases recovered after appropriate therapies. Chylous effusions (n=3) with the typical macroscopical and cytological appearance,[9] and associated with cardiological abnormalities. Modified transudates (n=3) that in two cases were associated with

intra-abdominal tumors evidenced at necropsy, and in one case was diagnosed in a cardiopathic cat on which the treatment led to the remission of clinical signs, including effusions.

Repeatability and linearity under dilution

~~Results of repeated testing on the two effusions from cats with FIP and on the two "non FIP" effusions (a reactive/inflammatory and a neoplastic effusions) are reported in table 1, along with the results of linearity under dilution test~~.

As shown in the ~~table~~supplementary table S1, repeatability of samples with normal ΔTNC was better for both the DIFF and BASO counts as well as for the ΔTNC, with CVs lower than 2.56%. Conversely, CVs were higher and extremely variable for the samples with high ΔTNC, due to a high variability of both BASO and DIFF counts which in turn induced a high variability of the ΔTNC.

Linearity under dilution provided excellent results for the TNCC-DIFF and TNCC-BASO of the sample with normal ΔTNC, with correlation coefficients of 0.99 and 1.00, respectively (P<0.001). Consequently, the ΔTNC remained constant over the different dilutions and did not correlated with the values expected after dilution (r = 0.81; P=0.390) (~~figure 3~~supplementary figure S1), Conversely, the linearity under dilution of samples with high ΔTNC was satisfactory only for the DIFF-TNCC (r = 0.98; P=0.001) while the DIFF-BASO did not show the expected decrease of value and basically provided similar results independently on the dilution (r=0.02; P=0.825). Consequently, the ΔTNC decreased in a linear manner (r = 0.98; P = 0.001) as the dilution increased (~~Figure 3~~s~~S~~upplementary figure S1).

Comparison of TNCC-DIFF, TNCC-BASO and ΔTNC between cats with and without FIP (figure ~~4~~2)

The ΔTNC was significantly higher (P<0.001) in cats with FIP (~~mean ± SD: 12.5 ± 11.2;~~ median: ~~8.16~~9.3; min-max: 0.5-36.4) than in non-FIP cats (~~1.1 ± 0.4;~~ 1.0; 0.5-2.5).The TNCC- BASO and the TNCC-DIFF counts were significantly lower (P<0.001 and P<0.05, respectively) in cats with FIP (TNCC-BASO = ~~0.5 ± 1.1;~~ 0.2; 0.0-5.3; TNCC-DIFF = ~~4.5 ± 7.4;~~ 1.5~~3~~; 0.1-26.3) than in non-FIP cats (TNCC-BASO = ~~43.5 ± 127.0;~~ 10.1; 0.0-707.9. TNCC-DIFF = ~~52.6 ± 164.6;~~ 9.1; 0.1-~~34.69~~21.8). Results from these latter cats were characterized by a high individual variability, likely due to the heterogeneity of the diseases responsible for the effusions. All the cats with FIP had a ΔTNC higher than 3.0 (the cut-off suggested by the previous study[12~~5~~]), except for the two cats which had "atypical" FIP see above) that had a ΔTNC of 0.538 for cat # 5, that had low SG, protein content and cellularity in the effusion associated with hemorrhagic foci in tissue, and 1.165 for cat # 25 that had a pericardial effusion cytologically characterized by large mesothelial cells and cytophagia. All the non-FIP cats had a ΔTNC lower than 3.0. More specifically, only 2 samples from cats without FIP had a ΔTNC higher than 1.7. These latter were a case of lymphoma with a high cellularity (25.45 cells x 10^3/µL according to the TNCC-DIFF), and the other was a modified transudate from a cardiopathic cat that was almost acellular in both the TNCC-DIFF (0.05 cells x 10^{9}/~~µ~~L) and in the TNCC-BASO (0.02 cells x 10^{9}/~~µ~~L).

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Diagnostic accuracy of the ΔTNC

The area under the ROC curve for the ΔTNC (figure ~~5~~3) was 0.9~~45~~ (95% C.I. = 0.84-1.00) (P<0.001 compared with the line of no discrimination). The best cut-off determined by the ROC curve analysis was 1.7. At this value ~~(~~Sens was ~~:~~ ~~92~~90.0% (95% C.I. = 68.3-98.8%), ~~;~~Spec was~~:~~ ~~93.5~~93.5% (95% C.I. = 78.6-99.2%),~~;~~ LR+ was ~~: 14~~13.9 (95% C.I. = 4.6-86.3),~~)~~ and LR- was 0.1 (95% C.I. = 0.0-0.3). ~~-~~The specificity increased to 100% using a cut-off of 2.5.

Discussion

The diagnosis of FIP should be based on a combination of clinical and laboratory findings. ~~and actually there are no tests that, taken alone, are able to confirm the diagnosis in vivo~~.[2] ~~However,~~ T~~t~~he analysis of effusions, when present, is a useful tool to support a clinical diagnosis of FIP or, conversely, to diagnose a different disease and~~, ultimately,~~ rule out FIP from the list of possible diagnoses.[1,5,18~~0~~] ~~In turn, the analysis of effusions includes a series of observations such as the macroscopic analysis of the fluid, physico-chemical (specific gravity, protein content, cell count) and/or cytological analysis, etc. Among these tests, the quali-quantitative evaluation of proteins contained in the effusion may have a diagnostic relevance since~~ .FIP, differently from other diseases characterized by protein rich effusions,[9,18~~0~~] is characterized by effusions containing a large amount of globulins and, in particular, of γ-globulins[7,~~2~~19~~1~~] and fibrinogen~~.~~[5] that react with acidic solution in the Rivalat's test and clot into the tube. ~~This latter is the main responsible for the formation of fibrin clots that may be observed macroscopically, and for the presence, in cytological specimens, of the granular eosinophilic background that strongly supports the diagnosis of FIP.[9,10] These proteins precipitate in acidic solutions, providing positive results in the Rivalta's test, i.e. the formation of jellyfish like clouds of proteins after placing a drop of fluid in water added with acetic acid.[2]~~ Recently, the Rivalta's test has been found to be highly diagnostic for FIP, although, as any other test, its specificity and sensitivity are not ~~absolute~~100%~~.~~[10~~3~~]~~–~~. In the present study it has been investigated whether cell counts in the laser-based counter Sysmex T-2000iV, that has the so-called BASO channel on which cells are counted after precipitation in an acidic reagent, can have diagnostic performances similar to those of the Rivalta's test, as suggested by a previous study that, however, included only a few samples of effusions from cats with FIP.[12~~5~~] To this aim a larger group of cats with FIP has been examined and the results have been compared with those obtained from cats with other diseases. A strict inclusion criteria has been applied, especially for the FIP group, on which have been included only cats with the disease confirmed by necropsy ~~or by a complete clinico-pathological screening that included cytology and protein analysis of the effusions, serum protein electrophoresis and serum concentration of AGP, that it has been demonstrated to be the~~

~~more reliable tool to support a diagnosis of FIP in challenging cases[4] or when the pre-test probability of FIP is elevated as for the cats included in the present study.[3]~~. Unfortunately, the "non FIP" group was composed largely by neoplastic effusions, that ultimately are not a challenging differential diagnosis for FIP, since the two conditions (tumor and FIP) may be easily differentiated through cytology. Therefore, a possible limitation of this study is the low number of non-neoplastic effusions that in routine practice may benefit from an additional test to address the diagnosis towards FIP rather than other types of inflammatory or reactive effusions. However, also in the previous study on effusions[12] all the samples from cats with inflammatory effusions other than FIP had a ΔTNCC lower than 1 confirming that a ΔTNCC higher than 1 has a high diagnostic accuracy for FIP . ~~Due~~a ~~to the retrospective nature of our study, it was impossible to form a group of inflammatory effusions large enough to be statistically compared with the group of FIP cats.~~

The results of the current study confirmed that the instrumental analysis with the Sysmex counter may be a further reliable approach to the analysis of FIP fluids. Specifically, since cells are entrapped in clots formed by fibrinogen precipitation in the BASO channel~~, as demonstrated by the previous study cited above,[15]~~ cell counts in the BASO channel (BASO-TNCC) are usually lower than those of the DIFF channel~~, the other channel used by the instrument for nucleated cell counts~~. This mechanism explains why the ΔWBC (in this study referred as ΔTNC) increases in such samples. Based on our results, the ΔTNC has a high diagnostic accuracy for FIP since, at the cut-off that according to the ROC curve analysis maximizes sensitivity and specificity, both these values were higher than 90%, with a positive likelihood ratio ~~higher than~~close to 14, indicating that if the ΔTNC value is higher than 1.7 it is 14 times more likely that the effusion comes from a cat with FIP than that the effusion comes from a cat with a different disease and a negative likelihood ratio of 0.1 indicating that if the ΔTNC value is lower than 1.7 the probability that the effusion comes from a cat with FIP is about 10% compared with the probability that the effusion comes from a cat with a different disease. The specificity becomes ~~absolute~~ equal to 100% if the ΔTNC is higher than 2.5,

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a value that was ultimately found in all the cats with FIP, except 2 cats which had atypical effusions. ~~that did not allow an easy identification of FIP even by using more "traditional" approaches, such as cytology and protein analysis~~. Specifically, the effusion in one cat with hypoalbuminemia and an hemorrhagic syndrome was classified as a transudate~~, due to its low specific gravity and protein content~~.[2] ~~Transudates may depend on hepatic failure, that may induce hypoalbuminemia and hemorrhagic syndromes due to a decreased production of clotting factors, including fibrinogen. Unfortunately, no additional tests to assess liver function were performed in this cat but hypoalbuminemia and hemorrhagic syndromes were present. Therefore,~~All these changes are consistent with liver failure that may induce also hypofibrinogenemia ~~may have also been present and both the precipitation of proteins in the cytological specimen~~ and therefore the clotting in the BASO reagent did not occur. In the other case the cytological pattern of the effusion was complicated by the presence of ~~large and somewhat~~ "atypical" mesothelial cells that usually in FIP effusions are less abundant than neutrophils and lymphocytes~~, but that in this case were the prevalent population~~. However, similar cells, often leading to a false diagnosis of neoplasia, may be frequently found in pericardial effusions.[9] ~~as in this case.~~ Therefore, in both cases the "false negative" results of the ΔTNC may depend on atypical features of the FIP fluid rather than on the low analytical sensitivity. As regards specificity, only two "false positive" results were found: one case of lymphoma, which in people sometimes provides positive Rivalta's test results,[1][4] possibly due to the presence of fibrinogen associated with an inflammatory reaction against the tumor itself~~ (not assessed in our case by acute phase protein measurement or serum protein electrophoresis),~~ one case with a poorly cellular fluid, on which the high ΔTNC is clearly a "mathematical artifact" due to analytical sensitivity of the instrument. Both these cases, however, do not represent a diagnostic challenge in routine practice, since FIP may be easily excluded if additional investigation are added to the diagnostic approach. ~~, such as the cytology of the fluid or clinical investigation and diagnostic imaging.~~

Independently on these few cases, the analysis of effusions with the Sysmex-XT2000iV counter evidenced a sensitivity and a specificity comparable to or even higher than that previously reported for the Rivalta's test,[10] likely because the mechanisms responsible for the Rivalta's test positivity and for the high ΔTNC are very similar. The Rivalta's test is rapid, cheap and accurate but it may suffer from some preanalytical or analytical factors. For example, the test may be inaccurate if inappropriate techniques are used or due to intrinsic factors of the reagents (e.g. concentration of acetic acid, different temperature between the fluid sample and the acetic acid solution, high pH of the reagent).[8,11,14] Additionally, the Rivalta's test provides semi-quantitative results (negative, weakly or strongly positive) and does not allow to grade the severity of the change. Finally, the evaluation of the test is subjective and no information about inter-observer variability are available. Conversely, the analysis with the Sysmex counter is more standardized in terms of reagents, although the repeatability study demonstrated that, limited to FIP effusions, it may suffer from a poor precision, that however did not affect the interpretation of the results, since values were always largely higher than 1.0. Moreover, the test is rapid and, ~~it provides~~ in a single measurement ~~both the~~it suggest the presence ~~estimate~~ of precipitable proteins (as the Rivalta test), a provisional information on the cell types, that may be estimated through the analysis of the scattergram[12,15] and the cell count. On this regard, 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, that 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 is generated by the BASO channel. Conversely, when FIP is clinically suspected, it may be recommended to directly check the results of the DIFF-TNCC and the ΔTNC that are reported in the Service screenshot of the software. Moreover, it may be interesting, in the future, to assess whether other laser-based instruments such as those of the ADVIA series, that uses a similar analytical principle to count basophils in peripheral blood[15,18] provide the same interesting results on FIP effusions.

Conclusion

In conclusion, this test evidenced a very high diagnostic accuracy of the ΔTNC for the diagnosis of FIP. This depends on the formation, in the BASO reagent, of clots that entrap the cells, similarly to what occurs in the Rivalta's test, that has also been reported to have a high diagnostic accuracy for FIP. This reaction leads to a low BASO-TNCC even when DIFF-TNCC counts are high. Therefore, in routine practice, it is not recommended to use the default TNCC counts generated by the BASO channel, but to directly use the DIFF-TNCC and especially the ΔTNC, particularly when FIP is suspected. In these cases, a ΔTNC higher than 1.7 strongly increases the probability of FIP, and a ΔTNC higher than ~~2.5~~3.4 may be considered a~~n almost~~ conclusive test to diagnose FIP.

Conflict of interest statement

The Authors do not have any conflict of interest potentially influencing the results of this study

Acknowledgments

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Supplementary Table S1: Results regarding repeatability recorded in two cats with high ATNC associated with FIP and in two cats with normal ATNC

	Repeatability											
	Cat #1 (FIP)			Cat # 2 (FIP)			Cat # 2 (lymphoma)			Cat # 4 (Inflammation)		
	DIFF	BASO	ATNC	DIFF	BASO	ATNC	DIFF	BASO	ATNC	DIFF	BASO	ATNC
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**
	Linearity under dilution											
	Cat #1 (FIP)			Cat # 2 (FIP)			Cat # 2 (lymphoma)			Cat # 4 (Inflammation)		
	DIFF	BASO	ATNC	DIFF	BASO	ATNC	DIFF	BASO	ATNC	DIFF	BASO	ATNC

Undiluted	nd	nd	nd	0.97	0.11	8.818	5.67	5.53	1.025	nd	nd	nd
50%	nd	nd	nd	0.48	0.1	4.800	2.54	2.54	1.000	nd	nd	nd
25%	nd	nd	nd	0.2	0.11	1.818	1.05	1.16	0.905	nd	nd	nd
12.5%	nd	nd	nd	0.21	0.1	2.100	0.67	0.73	0.918	nd	nd	nd
6.25%	nd	nd	nd	0.12	0.12	1.000	0.34	0.4	0.850	nd	nd	nd

nd = not determined

Figure captions

Figure 1: flow diagram summarizing the inclusion and exclusion criteria applied during the selection of cases from the database and the final composition of the study groups.

~~Figure 2: Pathological and cytological findings of the two cats with atypical effusion (A and B: cat #5, that had an effusion with low proteins, low specific gravity and poorly cellular; C and D: cat #25 that had atypical cytological findings). Cat # 5 had multifocal to coalescing subserosal fibrinous foci typical of FIP, on which, however, hemorrhages were found as in the example in A that shows the foci on the intestinal wall. Histology of these lesions was consistent with the diagnosis of FIP and intralesional FCoVs were detected by immunohistochemistry (B, 100 X magnification, ABC method, Mayer hematoxylin counterstain); the pericardial effusion from cat # 25 was characterized by the presence of numerous large round to pleomorphic cells, characterized by a severe anisocytosis and anisokaryosis, with abundant weakly basophilic cytoplasm, sometimes in cytophagia (C, 1000 X magnification, May Grünwald Giemsa). The presence of an evident brush border and the morphology supports the mesothelial origin of these cells. Other findings potentially consistent with FIP were less evident: neutrophils and lymphocytes were numerically less abundant than mesothelial cells and the proteinaceous background was very weak. However, necropsy evidenced fibrinous pleuritis and pericarditis and histology / immunohistochemistry confirmed the diagnosis of FIP and the presence of intralesional FCoVs (D, 100 X magnification, ABC method, Mayer hematoxylin counterstain)~~

~~Figure~~ ~~Supplementary figure S13~~: ~~Linearity under dilution (LUD) recorded in serially diluted effusion samples from a cat with lymphoma (A, B, C) and in a cat with FIP (D, E, F). Data regarding absolute values of TNCC-DIFF, TNCC-BASO and ATNC of the two undiluted samples are reported in table 1. The solid line indicates the linear correlation between expected and observed~~

~~values expressed as percentage of the result of the undiluted sample; dotted lines indicate the 95% Confidence Interval (CI). Observed values were statistically correlated with the expected value according to a linear model for the TNCC-DIFF (A) and for the TNCC-BASO (B) of the cat with normal ΔTNC affected by lymphoma. In this cat the ΔTNC did not decrease along with the dilution of the sample (C). Conversely, in the cat with FIP, only the TNCC-DIFF (D) but not the TNCC-BASO (E) statistically correlated with the expected value according to a linear model. Consequently, the ΔTNC (F) decreased in diluted samples and was significantly correlated with the magnitude of dilution.~~

Figure ~~4~~2: Values of TNCC-DIFF (A), TNCC-BASO (B) and ΔTNC (C) recorded in cats with FIP and in cat with diseases other than FIP (Non FIP). The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median, whiskers extend to further observation within the I quartile minus 1.5*IQR or to further observation within the III quartile plus 1.5*IQR. ~~Near outliers are indicated by the orange symbols "+" and far outliers with an orange asterisk~~Dots indicates the values recorded in this study. The TNCC-DIFF and the TNCC-BASO graphs do not include the result of a neoplastic (Non-FIP) sample that had an extremely high TNCC-DIFF and TNCC-BASO count (921.8 and 707.9 cells x 10^9/L). The black bolded asterisks reported ~~in the boxes~~below the X axis indicate significant differences between groups (* = P<0.05; *** = P<0.001).

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Figure ~~5~~3: Receiver operating characteristic (ROC) curves of the ΔTNC for the diagnosis of FIP. The gray line indicates the line of no discrimination.

Supplementary material

Supplementary figure S1: Linearity under dilution (LUD) recorded in serially diluted effusion samples from a cat with lymphoma (A, B, C) and in a cat with FIP (D, E, F). Data regarding absolute values of TNCC-DIFF, TNCC-BASO and ΔTNC of the two undiluted samples are reported in table 1. The solid line indicates the linear correlation between expected and observed values expressed as percentage of the result of the undiluted sample; dotted lines indicate the 95% Confidence Interval (CI). Observed values were statistically correlated with the expected value according to a linear model for the TNCC-DIFF (A) and for the TNCC-BASO (B) of the cat with normal ΔTNC affected by lymphoma. In this cat the ΔTNC did not decrease along with the dilution of the sample (C). Conversely, in the cat with FIP, only the TNCC-DIFF (D) but not the TNCC-BASO (E) statistically correlated with the expected value according to a linear model. Consequently, the ΔTNC (F) decreased in diluted samples and was significantly correlated with the magnitude of dilution.

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Supplementary Table S1: Results regarding repeatability recorded in two cats with high ΔTNC associated with FIP and in two cats with normal ΔTNC

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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**
Linearity under dilution												
	Cat #1 (FIP)			Cat # 2 (FIP)			Cat # 2 (lymphoma)			Cat # 4 (Inflammation)		
	DIFF	BASO	ΔTNC	DIFF	BASO	ΔTNC	DIFF	BASO	ΔTNC	DIFF	BASO	ΔTNC

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Undiluted	nd	nd	nd	0.97	0.11	8.818	5.67	5.53	1.025	nd	nd	nd
50%	nd	nd	nd	0.48	0.1	4.800	2.54	2.54	1.000	nd	nd	nd
25%	nd	nd	nd	0.2	0.11	1.818	1.05	1.16	0.905	nd	nd	nd
12.5%	nd	nd	nd	0.21	0.1	2.100	0.67	0.73	0.918	nd	nd	nd
6.25%	nd	nd	nd	0.12	0.12	1.000	0.34	0.4	0.850	nd	nd	nd

nd = not determined

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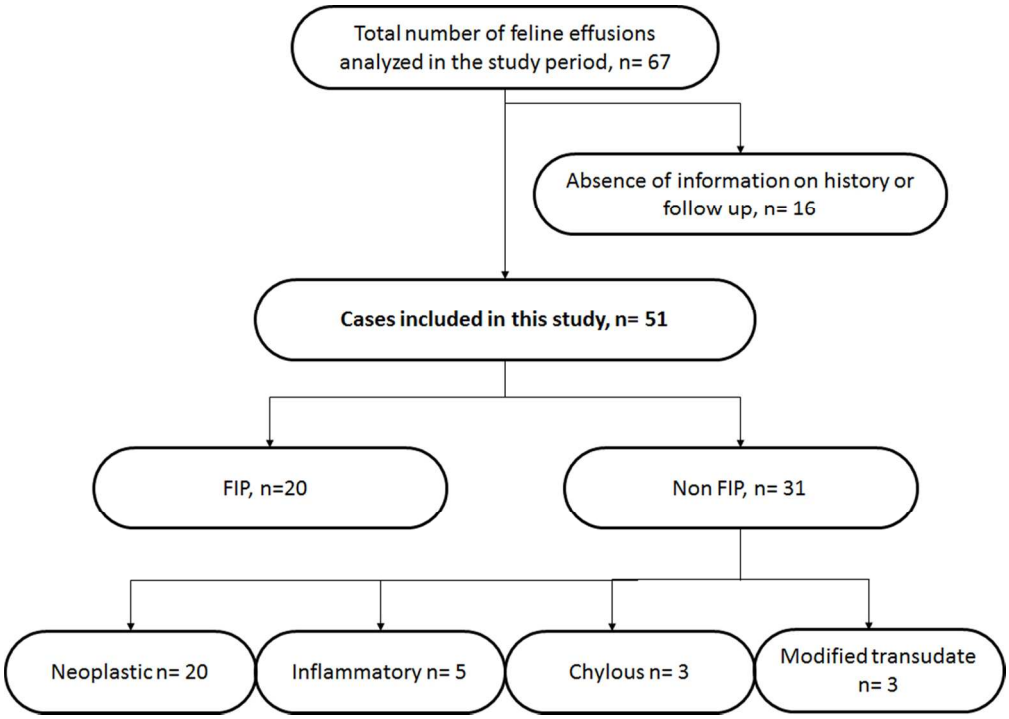

Figure 1: flow diagram summarizing the inclusion and exclusion criteria applied during the selection of cases from the database and the final composition of the study groups.
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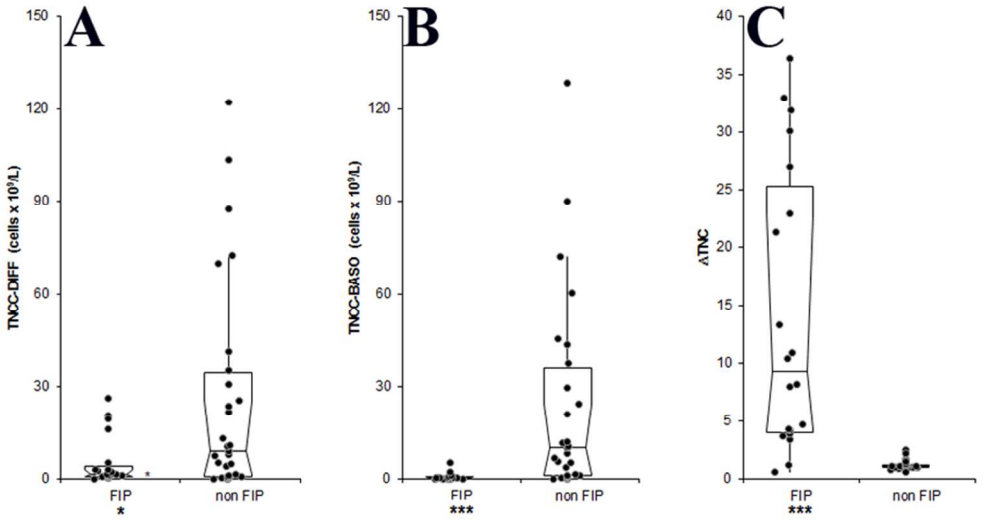

Figure 2: Values of TNCC-DIFF (A), TNCC-BASO (B) and ΔTNC (C) recorded in cats with FIP and in cat with diseases other than FIP (Non FIP). The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median, whiskers extend to further observation within the I quartile minus 1.5*IQR or to further observation within the III quartile plus 1.5*IQR. Dots indicates the values recorded in this study. The TNCC-DIFF and the TNCC-BASO graphs do not include the result of a neoplastic (Non-FIP) sample that had an extremely high TNCC-DIFF and TNCC-BASO count (921.8 and 707.9 cells x 10^9/L). The black bolded asterisks reported below the X axis indicate significant differences between groups (* = P<0.05; *** = P<0.001).
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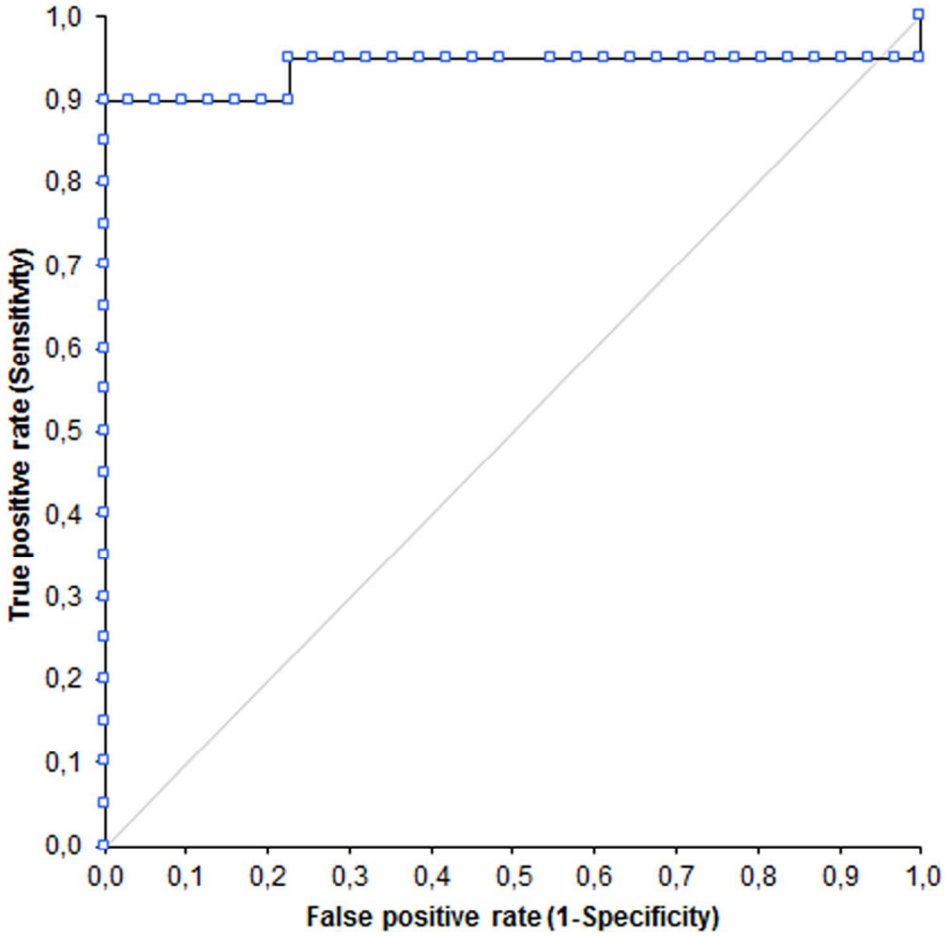

Figure 3: Receiver operating characteristic (ROC) curves of the ΔTNC for the diagnosis of FIP. The gray line indicates the line of no discrimination.
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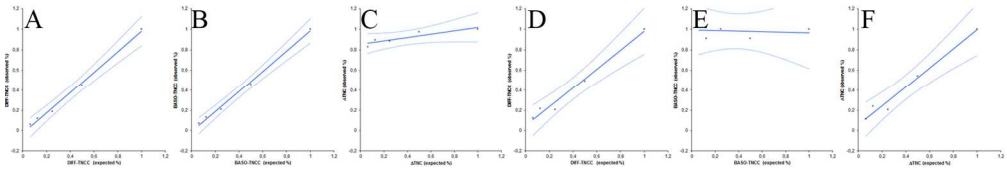

Supplementary figure S1: Linearity under dilution (LUD) recorded in serially diluted effusion samples from a cat with lymphoma (A, B, C) and in a cat with FIP (D, E, F). Data regarding absolute values of TNCC-DIFF, TNCC-BASO and ΔTNC of the two undiluted samples are reported in table 1. The solid line indicates the linear correlation between expected and observed values expressed as percentage of the result of the undiluted sample; dotted lines indicate the 95% Confidence Interval (CI). Observed values were statistically correlated with the expected value according to a linear model for the TNCC-DIFF (A) and for the TNCC-BASO (B) of the cat with normal ΔTNC affected by lymphoma. In this cat the ΔTNC did not decrease along with the dilution of the sample (C). Conversely, in the cat with FIP, only the TNCC-DIFF (D) but not the TNCC-BASO (E) statistically correlated with the expected value according to a linear model. Consequently, the ΔTNC (F) decreased in diluted samples and was significantly correlated with the magnitude of dilution.
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