Title: Use of hyaluronidase to improve analysis of feline body cavity effusions

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Short running title: Use of hyaluronidase in analysis of feline cavity effusions

Abstract

Classification of body cavity effusions is an important step in the investigation and diagnosis of disease in cats. Feline inflammatory effusions are often highly proteinaceous and viscous, which can cause clumping of white cells and subsequently inaccurate nucleated cell counts using automated and manual methods. Microscopic assessment of cellularity can also be difficult due to the varying thickness of smears and cell clumping which skews white cell distribution. The Advia 120 uses 2 white cell counting channels, the basophil/lobularity and differential/peroxidase channels which can provide quite different results in highly viscous feline samples and often disagree with smear assessment of cellularity. We investigated the effects of pre-incubation of feline effusion samples with hyaluronidase and its effects on nucleated cell counts and cellularity assessment. Nucleated cell counts were obtained by both automated analysis using the Advia 120 and manual counting methods. Agreement was assessed using a Bland-Altman chart. Pre-treatment of samples with hyaluronidase resulted in good agreement between the Advia basophil channel and manual counting methods in all samples in the study. However, improvements in nucleated cell counts after hyaluronidase treatment were significantly greater in clumped samples and cell distribution of these samples on direct smears was also improved. Therefore, when nucleated cell clumping is observed on a direct smear, pre-treatment of the sample with hyaluronidase prior to analysis on an automated analyzer is advised with the WBC/baso channel the most accurate nucleated cell counting channel.

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Keywords

Automated analysis, feline effusion, hyaluronidase, nucleated cell counts

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Classification of body cavity effusions is an important step in the investigation and diagnosis of disease as it can give an indication of the underlying mechanism causing the abnormal fluid accumulation.[8] Classification is achieved by measuring the protein concentration and the nucleated cell count (NCC) of the effusion. Nucleated cell counts are normally measured by using an automated hematology analyzer or manually by hemocytometer. However obtaining accurate NCCs from feline effusions is often problematic due to their high viscosity and the tendency for white cell clumping, and previous studies have indicated only moderate correlation between automated and manual methods when analysing fluid samples from this species.[6]

Manual cell counting using a hemocytometer is the traditional method for obtaining fluid NCC. However this method is time consuming and possesses inherent error.[11] Clumping of cells can also contribute to inaccurate cell counts using this method.[7] Automated hematology analyzers in contrast provide rapid results that are less labor intensive to obtain compared to traditional cell counting methods. These analyzers also provide more accurate NCCs however provided no nucleated cell clumping is present.[11] The Advia 120[a] hematology analyzer measures white cells by 2 methods: the basophil/lobularity (WBC/baso) and the differential/peroxidase (WBC/perox) channel. The WBC/baso channel provides a total NCC; the WBC/perox channel provides both a differential NCC and a total NCC. Large discrepancies are often found between these 2 Advia 120 channels when analyzing feline effusions and this has also been reported when analysing feline effusions using the Sysmex XT-2000i.[5] In our experience, examination of a direct smear of an effusion can indicate that neither NCC is accurate however microscopic assessment of the NCC from the direct smear is complicated by the thick nature of these smears and cell clumping, which skews the white cell distribution.

Problems with obtaining accurate NCCs have been encountered with other fluids that are of high viscosity. In synovial fluids, this has been overcome by pre-incubation of samples with hyaluronidase prior to analysis by automated analyzers.[3,9] This technique has also been demonstrated to have utility in forensic medicine when analysing vitreous humor.[4] The viscous nature of synovial fluid is caused by high levels of hyaluronic acid.[10] Incubation with hyaluronidase causes the hydrolysis of hyaluronan and this decreases the viscosity of the sample. Hyaluronic acid has been shown to be increased in induced cases of peritonitis in rabbits[2] and it is possible that this mechanism may contribute to the high viscosity of feline inflammatory body cavity effusions. We therefore hypothesized that pre-incubation of feline body cavity effusions with bovine testicular hyaluronidase will decrease the viscosity of the samples allowing more accurate NCC results by automated hematology analyzers and better distribution of cells on direct smears making assessment of cellularity easier.

To test this hypothesis, feline body cavity effusions submitted to the clinical pathology laboratory of the University of Glasgow were analyzed both with and without pre-incubation with hyaluronidase. The effusions were submitted to the laboratory in EDTA tubes and were analyzed on the day of arrival. Direct smears of the untreated samples were made and stained using the May-Grünwald Giemsa method. These smears were examined to determine cell distribution and the presence or absence of cell clumping.

Automated NCCs were then obtained using the Advia120 and manual NCCs obtained by using a Neubauer counting chamber. To perform the manual NCC, 20µL of sample was added to 380 µL of 1% acetic acid in order to lyse erythrocytes (methyl violet was added to stain the nuclei). The sample was rotated gently for 2-3 min and then transferred to the Neubauer counting chamber using a capillary tube. The counting chamber was left in a moist chamber for 10 min to let cells settle before cell counting using a light microscope.[1]

After initial analysis of the untreated sample, a 250 µL aliquot of sample was added to 250 µL of hyaluronidase (150 U/mL) and incubated at 37 °C for 10 min. The hyaluronidase solution of 150 U/mL had been prepared by dissolving 10 mg of bovine testicular hyaluronidase[b] solution (439 units/mg) in 30 mL of saline buffer (0.9 g/L). After 10min of incubation with hyaluronidase, the treated sample was run through the automated hematology analyzer (Advia 120) to obtain automated NCCs. Manual NCCs on the treated sample were performed as described above. Results obtained were then corrected for the dilution caused by hyaluronidase added to the sample. A direct smear was also made immediately after incubation to assess white blood cell distribution and presence of clumping. Samples were thoroughly mixed in all instances before performing any procedures.

To demonstrate the action of hyaluronidase and confirm that any changes in the results obtained were not caused solely to dilution, an equal amount of normal saline was added to an aliquot of untreated sample. The mixture was incubated at 37°C for 10 min and NCCs obtained using the Advia120 and manually with the hemocytometer.

Fluid protein measurements on untreated samples were obtained using the Biuret method on a Olympus AU640 analyzer[c].

Results from the Advia 120 and manual counts were compared using Pearson's correlation (r) and Bland-Altman plots. Fluid protein levels in clumped and non-clumped samples were compared using Student's t-test. The manual NCC obtained from hyaluronidase treated samples was considered the benchmark result as the presence or absence of clumping could be ascertained at the time of analysis. Statistical significance was established at ($p<0.05$). Data were analyzed using a spreadsheet software package[d].

Repeatability of NCC from the Advia120 in untreated samples was assessed on 2 samples with no obvious nucleated cell clumping and 1 sample with clumping observed by

collecting 5 consecutive measurements of each on the same day and then calculating the coefficient of variation (CV) with the formula CV = standard deviation (SD)/mean x100.

Precision of the analyzer was found to be adequate when analyzing non-clumping samples, with WBC/baso channel CVs <5%. However the CVs from the sample where clumping was present were much greater (see Table 1).

In total, 25 feline body cavity effusions were submitted to the diagnostic service during the time of the study (13 peritoneal, 12 pleural), and counts were obtained as noted above (Table 2).

Smears made from untreated samples were assessed for white cell clumping and this phenomenon was noted in 13/25 samples (9 peritoneal, 4 pleural,Table 2). Smears prepared from hyaluronidase-treated samples showed improved white cell distribution with no cell clumping. This allowed easier assessment of cellularity (taking into account the dilution factor) by microscopy.

Nucleated cell counts from untreated samples obtained from both the WBC/baso channel and WBC/perox channel showed moderate correlation with NCCs obtained from treated samples counted by the manual method ($r = 0.68$ and $r = 0.84$ respectively). These results are similar to a previous study of body cavity effusions from different animal species, which found only moderate correlation ($r = 0.73$) between cells counts obtained from the Advia 120 WBC/baso channel and the standard hemocytometer method in feline peritoneal effusions.[6] The Bland-Altman agreement charts showed that both the WBC/baso and WBC/diff channels underestimated the NCCs and this error was greater in samples where cell clumping was observed. Furthermore, the presence of cell clumping was more common in samples with higher cell counts (Fig. 1A and 1B).

After hyaluronidase treatment, greater agreement between the WBC/baso channel and the standard hemocytometer method was shown in both clumping and non-clumping samples

149 treated with hyaluronidase (Fig. 1C), however the magnitude of improvement was
150 significantly greater in the clumped samples (Student's *t*-test, $p<0.01$). The most reliable
151 NCC obtained from the automated analyzer in the treated samples was obtained from the
152 WBC/baso channel as results showed good correlation with the manual cells counts
153 (Pearson's correlation coefficient of $r = 0.99$). As greater agreement was seen in both
154 clumping and non-clumping samples, it could be argued that treating all feline effusions with
155 hyaluronidase prior to analysis would be beneficial. However, as the cellularity of samples
156 with no clumping tended to be low and the increase in NCC post-treatment small,
157 hyaluronidase incubation of samples with no clumping is unlikely to lead to significant
158 changes in the classification or interpretation of results. Clumped samples had statistically
159 significant greater fluid protein levels than non-clumped samples (Student's *t*-test, $p=0.024$)
160 however fluid protein was not predictive of the need for enzyme treatment as there was a
161 large overlap between the groups; many non-clumped samples had high fluid protein (Table
162 2). Enzymatic treatment should therefore be limited to samples where clumping is noted on
163 examination of a direct smear of the effusion.

164 In contrast to the WBC/baso channel, the NCCs from the WBC/perox channel
165 decreased after treatment and this resulted in the WBC/perox channel counts being markedly
166 low post-treatment when compared to results from the WBC/baso channel, the manual cell
167 count and visual assessment on a direct smear (see Table 2). The reason for the decrease in
168 the WBC/perox channel NCCs post-treatment is unclear particularly as smear assessment
169 demonstrated that white cell clumping in the samples had resolved after treatment with
170 hyaluronidase. Analysis of the WBC/perox scatter plot shows more events in the "noise"
171 region in treated samples and we hypothesize that enzyme treatment causes increased
172 fragility of the cells which then disintegrate in WBC/perox channel.

The results of this study are in contrast to the recommendations by Giordano et al.[5] who demonstrated high diagnostic accuracy of the ΔTNC (the ratio of the WBC/perox and WBC/baso counts) for diagnosis of FIP in feline effusions. This disparity in NCC between the WBC/perox and WBC /baso channels, they hypothesize, is due to the acidic WBC/baso reagent causing precipitation of proteins with subsequent entrapment of nucleated cells and low NCCs. Consequently, they advise use of the WBC/perox channel for nucleated cell counts in feline effusions. However, the samples in their study were not pre-treated with hyaluronidase. We demonstrate in Fig. 1B, that while the WBC/perox NCC is likely to be more accurate in untreated samples, if cell clumping is present the NCC values are likely to be underestimated by automated analyzers.

Dilution of the samples with an equal volume of saline did not improve the accuracy of the analyzer cell counts (data not shown). This along with the success of the hyaluronidase would support the hypothesis that substances such as hyaluronan contribute to the highly viscous nature of some of these effusions.

In summary, pre-treatment with hyaluronidase improved the NCCs obtained from the WBC/baso channel in all samples in the study. Greatest improvement was seen in samples where nucleated cell clumping was observed on a direct smear whereas samples with no clumping observed had only small improvements in NCC. Fluid protein was significantly higher in clumped samples but was not a good predictor for the requirement of enzyme treatment therefore the decision for enzyme treatment should be based on the presence of nucleated cell clumping. The WBC/diff channel NCCs were consistently decreased by treatment and therefore this channel was not reliable for determining accurate NCC after enzyme treatment. Finally, pre-treatment with hyaluronidase produced more even distribution of white cells on a direct smear allowing easier assessment of cellularity by microscopy.

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Sources and manufacturers

[a]Advia 120, Siemens, Frimley, Surrey, UK

[b]Bovine testicular hyaluronidase, Sigma-Aldrich, Irvine, Ayrshire, UK

[c]Olympus AU640, Beckman Coulter, High Wycombe, Buckinghamshire, UK

[d]Microsoft Excel 2010, Redmond, Washington, USA

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Tables

Table 1. Precision data for nucleated cell counts from untreated feline effusions using both Advia 120 WBC/baso and WBC/perox channels.

Sample	Advia channel	Mean ($NCCx10^9/L$)	SD	CV (%)
1*	WBC/baso	1.74	0.38	21.8
	WBC/perox	0.92	0.32	34.8
2	WBC/baso	2.24	0.07	3.12
	WBC/perox	1.23	0.24	19.5
3	WBC/baso	4.64	0.12	2.58
	WBC/perox	2.43	0.58	23.9

* = nucleated cell clumping present on the direct smear.

253 **Table 2. Total nucleated cell counts and fluid protein measurements.**

Sample no./site		Advia WBC/baso channel (x 10^9/L)	Advia WBC/Perox Channel (x 10^9/L)	Manual cell count (x 10^9/L)	Fluid Protein g/dL
1* Peritoneal	Untreated Hya treated	10.57 15.10	10.57 5.70	13.4	6.6
2 Pleural	Untreated Hya treated	42.44 48.74	37.10 18.48	43	3.0
3 Peritoneal	Untreated Hya treated	2.49 3.92	3.10 1.82	5.2	4.9
4* Pleural	Untreated Hya treated	4.78 10.60	7.63 6.40	10.9	4.1
5* Peritoneal	Untreated Hya treated	2.28 4.4	3.01 0.64	5.4	4.7
6* Peritoneal	Untreated Hya treated	9.25 11.06	1.67 0.56	11.8	3.2
7* Peritoneal	Untreated Hya treated	1.14 3.98	2.22 1.72	4	7.0
8 Pleural	Untreated Hya treated	1.21 1.50	0.43 0.52	1.9	3.7
9* Peritoneal	Untreated Hya treated	1.87 9.66	3.24 2.28	9.8	8.5
10 Pleural	Untreated Hya treated	13.50 12.60	9.77 5.22	14	5.2
11 Pleural	Untreated Hya treated	1.69 3.62	3.64 1.52	4.2	6.1
12 Peritoneal	Untreated Hya treated	4.59 4.84	3.37 2.8	4.6	4.4
13 Peritoneal	Untreated Hya treated	1.65 1.88	1.04 1.00	2.4	4.1
14* Pleural	Untreated Hya treated	88.85 241.36	88.85 32.88	202	5.7
15 Pleural	Untreated Hya treated	0.37 1.14	1.50 1.14	1.7	5.0
16 Peritoneal	Untreated Hya treated	0.32 1.46	0.72 0.76	1.3	4.1
17 Pleural	Untreated Hya treated	4.68 5.58	4.68 4.08	5.9	6.2
18 Pleural	Untreated Hya treated	0.76 0.98	0.39 0.36	0.8	2.4
19 Pleural	Untreated Hya treated	0.10 0.10	0.06 0.04	0.1	0.2
20* Pleural	Untreated Hya treated	3.17 17.72	8.67 6.42	17.7	7.5
21* Peritoneal	Untreated Hya treated	2.71 4.60	2.36 1.94	4.95	6.0

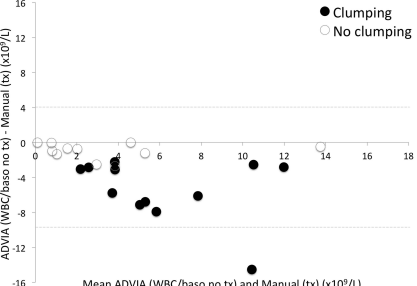
22* Peritoneal	Untreated Hya treated	1.48 9.28	7.03 3.18	8.6	4.1
23* Peritoneal	Untreated Hya treated	0.65 3.52	2.17 1.72	3.7	ND
24* Pleural	Untreated Hya treated	0.83 6.36	4.2 1.14	6.6	4.9
25* Peritoneal	Untreated Hya treated	1.91 9.56	3.59 2.62	8.7	5.6

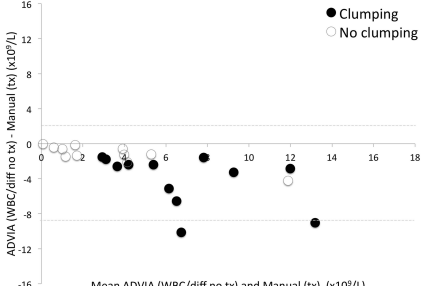
Total nucleated cell counts obtained from automated analyser channels WBC/baso and WBC/perox for both untreated and hyaluronidase treated samples and manual method using hyaluronidase treated samples with corresponding fluid protein measurements. * = white cell clumping observed on direct smears of untreated sample. ND = no data.

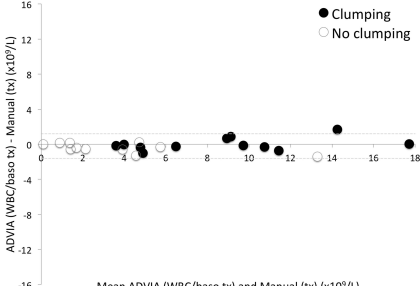
Figure legends

Figure 1A-C. Bland-Altman plots of NCCs obtained from feline body cavity effusions using the hemocytometer and the Advia 120 WBC/baso and WBC/perox channels. Agreement between the NCC obtained using hyaluronidase treated samples counted by the manual method (benchmark method) and untreated samples counted using the ADVIA 120 WBC/baso (plot A) and WBC/diff (plot B) channels are shown. Positive values on the y-axis indicate a higher reading with the automated method while negative values indicate a higher reading with the manual counting method. Both automated channels underestimate the NCC in untreated samples. Agreement between the NCCs of hyaluronidase treated samples using the Advia 120 WBC/baso and the manual method are shown in plot C. Good agreement is demonstrated between the manual method and the Advia 120 WBC/baso channel when counting treated samples. Two outliers with NCC >$40x10^9$/L were removed to improve graphical representation in the NCC range of interest.

Dashed lines represent ±2SD, no tx = no hyaluronidase treatment, tx = hyaluronidase treated samples.


Clumping
No clumping
ADVIA (WBC/baso no tx) - Manual (tx) (x10⁹/L)
Mean ADVIA (WBC/baso no tx) and Manual (tx) (x10⁹/L)
A


Clumping
No clumping
ADVIA (WBC/diff no tx) - Manual (tx) (x10⁹/L)
Mean ADVIA (WBC/diff no tx) and Manual (tx) (x10⁹/L)
B


Clumping
No clumping
ADVIA (WBC/baso tx) - Manual (tx) (x10⁹/L)
Mean ADVIA (WBC/baso tx) and Manual (tx) (x10⁹/L)
C