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Relationship between automated total nucleated cell count and enumeration of cells on direct smears of canine synovial fluid

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Abstract

Canine osteoarthritis is a common condition seen in veterinary clinical practice and causes considerable morbidity in dogs as they age. Synovial fluid analysis is an important tool for diagnosis and treatment of canine joint disease and obtaining a total nucleated cell count (TNCC) is particularly important. The low volume of fluid obtained during arthrocentesis is often insufficient for obtaining an automated TNCC, thereby limiting sample interpretation. The aim of the present study was to investigate whether estimation of TNCC in canine synovial fluid could be achieved by performing manual cell counts on direct smears of fluid.

Fifty eight synovial fluid samples, taken by arthrocentesis from 48 dogs, were included in the study. Direct smears of synovial fluid were prepared, and hyaluronidase added before cell counts were obtained using a commercial laser-based instrument. A protocol was established to count nucleated cells in a specific region of the smear, using a serpentine counting pattern; mean number of nucleated cells/400× field was then calculated. There was a positive correlation between the automated TNCC and mean manual cell count, with more variability at higher TNCC. Regression analysis was performed to estimate TNCC from manual counts. By this method, 78% of the samples were correctly predicted to fall into one of three categories (within the reference interval, mildly to moderately elevated, or markedly elevated) relative to the automated TNCC. Intra-observer and inter-observer agreement was good to excellent. The results of the study suggest that interpretation of canine synovial fluid samples of low volume can be aided by manual cell counting of direct smears.

Keywords

Automated cell counts; Dog; Joint fluid; Manual cell counts; Total nucleated cell counts

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Introduction

Orthopaedic disease is a frequent cause of morbidity in dogs. Synovial fluid analysis and determination of total nucleated cell count (TNCC) is important for the diagnosis and management of joint disease (MacWilliams and Friedrichs, 2003). However, a TNCC cannot always be obtained, when insufficient volume of synovial fluid is obtained (Johnson and Mackin, 2012) and this is particularly problematic in small dogs or in joints with limited effusion.

In the absence of an automated TNCC, synovial fluid analysis can be complicated, particularly when the TNCC is mildly increased and difficult to distinguish from a TNCC within the reference interval (WRI). Hemocytometers have been used to quantify cells in synovial fluid, but sources of error include unequal cell distribution and improper chamber filling, with an overall error reported to be typically 20% (Webb and Latimer, 2011). In one study, assessment of TNCC using a hemocytometer tended to underestimate the automated TNCC (Atilola et al., 1986). Estimation of TNCC by visual assessment of direct smears of synovial fluid has been attempted, although its viscous nature makes smear preparation and interpretation challenging (Fernandes, 2014).

We hypothesized that development of a Standard Operating Procedure for manual enumeration of nucleated cells in synovial fluid would generate values that accurately reflect the automated TNCC obtained using a laser-based analyzer. The objective of this study was to compare automated TNCC with manual counts after establishing a method for counting nucleated cells on direct smears of canine synovial fluid.

Materials and methods

Study design

This prospective study was conducted between July 2012 and May 2014 at the University of Wisconsin Veterinary Care Hospital. Canine synovial fluid samples, submitted to the Clinical Pathology Laboratory as part of routine diagnostic investigation, were recruited for the study. Inclusion criteria were that the specimen was not clotted, the specimen was of sufficient volume to make a direct smear and obtain an automated TNCC, a feathered edge was present on the smear, the base of the smear covered at least two-thirds the width of the slide, and the smear contained <10% ruptured nucleated cells in the region where counts were performed. Repeat samples of the same joint on a different day or samples from a different joint in the same dog were accepted. Informed owner consent for arthrocentesis was obtained as part of routine clinical practice. For each specimen, manual and automated cell counts were compared. Clinical diagnoses were obtained from the clinical records.

Processing of synovial fluid specimens

Synovial fluid samples, collected into potassium EDTA anticoagulant tubes (Microtainer, BD Diagnostics) were received and processed by the diagnostic laboratory. A direct smear of well-mixed fluid was made, without concentrating the cells, using two standard glass microscope slides at a 30–45 degree angle. Samples were not treated with powdered hyaluronidase before smear preparation, because it was determined in a pilot study that

correlation with automated TNCC improved without use of hyaluronidase. Smears were air-dried and stained with Wright-Giemsa stain (Wescor) using an automated stainer (Aerospray 7150 Hematology Slide Stainer-Cytocentrifuge, Wescor).

Automated TNCC

Automated TNCC were obtained using the Advia 120 Hematology Analyzer (Siemens Healthcare Diagnostics, multispecies software version 3.1.8-MS) (Aulesa et al., 2003), a laser-based system that requires 175 μL of sample. The performance specification for TNCC is $0.02\text{--}100 \times 10^3/\mu\text{L}$; precision for TNCC is mean $11.4 \times 10^3/\mu\text{L}$, SD $0.4 \times 10^3/\mu\text{L}$, and CV 3.5%. To facilitate obtaining an accurate automated TNCC, a small amount of powdered hyaluronidase (Hyaluronate glycanohydrolase lyophilized powder, 300 USP units/mg, Fisher Scientific) was added to the fluid to decrease its viscosity. The powdered hyaluronidase did not add appreciable volume to the sample. Specimens were mixed using an automatic mixer for at least 5 min (Ekman et al., 2010).

Manual counting procedure

Initially, all manual counting was performed by one individual (AD) blinded to the automated TNCC. The length of the smear was determined by observation at $40\times$ and $100\times$ magnifications. The start point was defined as the point closest to the label. If there was a distinct circular area where the drop of fluid was placed on the slide, the start point was where the smear began to spread width-wise from the drop; if the smear separated from the drop at different points on either side of the drop, the midpoint was chosen. The start point was marked on the lateral edge of the slide with a marker (mark 1; Fig. 1A). The end point of the smear was defined as the outermost tip of the feathered edge located at the opposite end of the smear. Thin lines of synovial fluid that trailed away from the main smear were excluded in making this judgment. The end point was marked on the lateral edge of the slide (mark 2; Fig. 1B). The length of the smear was measured in millimeters. A third mark was made on the lateral edge of the slide to mark the midpoint of the smear length-wise (mark 3; Fig. 1B).

Counting strategy used a serpentine pattern with nucleated cells in three rows of five fields (15 fields in all) counted in total (Fig. 1C). The field diameter was $550 \mu\text{m}$ (field area of 0.238 mm^2). Initially, the $40\times$ objective was centered on the mark at the midpoint of the smear (mark 3). The slide was moved across the width of the smear until the objective was estimated to be at the midpoint of the slide width. This placed the microscopic field in the third of the five counting fields in the first row (the middle field of the first row). The slide was moved two full microscopic fields toward the operator to the first counting field. Once the nucleated cells in the first field were counted, the slide was moved one field away from the operator and counting was repeated; all nucleated cells in a total of five adjacent fields in a row were counted. The slide was moved to the next row one field away from the feathered edge, and cells in an additional five adjacent fields were counted across the width of the slide. The slide was again moved one field away from the feathered edge and cells in a third row of five adjacent fields were counted.

All nucleated cells in a field were counted, including cells with pyknotic and karyorrhectic nuclei, cells in groups, and free nuclei from ruptured cells. To prevent duplicate counting, a cell was counted only if at least half the cell was within the microscopic field. The number of cells in each counting field was recorded. If nucleated cells were not present in a counting field, zero was recorded for that field. The mean nucleated cell count/field was calculated for each slide.

Intra- and inter-observer variation

To assess intra-observer variation, the observer who performed the initial manual cell counts (AD) performed a total of five manual counts on each of 12 slides (four from each of three different categories of TNCC) selected at random. The three categories of TNCC were selected for clinical relevance: WRI ($<3000/\mu\text{L}$) (MacWilliams and Friedrichs, 2003; Ekmann et al., 2010), mild to moderately elevated cell count ($3000\text{--}20,000/\mu\text{L}$) and markedly elevated cell count ($>20,000/\mu\text{L}$). Four new observers, blinded to slide identity and TNCC values, performed counts once on each of the same 12 slides. Marks were removed from the slides between observers, who included one clinical pathologist, one clinical pathology resident, one research laboratory technician with limited microscopy experience, and one student researcher with no experience evaluating canine cytological samples. All observations were performed using the same microscope. To assess reliability of smear preparation, five specimens with a range of TNCC were selected and five smears were prepared from each one. A manual cell count was determined for each slide by a blinded observer (AD).

Statistical analysis

Statistical analysis was performed using R (R Foundation for Statistical Computing), Stata, (StataCorp), Graphpad Prism 6.0c and the MH Program version 1.2.¹ Using the Shapiro-Wilk normality test, automated TNCC and mean field count data were found not to approximate a normal distribution. Passing Bablok regression was performed to examine the relationship between the automated TNCC measurement and the mean nucleated cell count/400 $\times$ field on smears. Correlation between the two methods was examined using the Spearman rank statistic. A Bland-Altman plot of TNCC difference (Estimated TNCC – Automated TNCC) versus TNCC average plot was also constructed. Actual and predicted TNCC were categorized in a contingency table of counts as WRI, mildly to moderately increased, or markedly increased. The McNemar's and the Bowker symmetry tests were used to examine marginal homogeneity within the contingency table. Results were considered significant at $P < 0.05$. Intra-observer and inter-observer repeatability in estimated TNCC by manual cell counting was examined using the intra-class correlation coefficient statistic (ICC). Reliability of smear preparation for estimation of TNCC by manual counting was examined using the ICC statistic. ICC cutoffs were defined as follows: <0.40 , poor agreement; $0.41\text{--}0.60$, moderate agreement; $0.61\text{--}0.79$ good agreement; and >0.80 , excellent agreement (Landis and Koch, 1977).

¹See: <http://john-uebersax.com/stat/mh.htm>

Results

Sample population

A total of 58 samples met the inclusion criteria. Specimens were collected from 54 joints in 48 dogs. Ages ranged from 64 days to 10.5 years, with a median age of 5.7 years. The breeds represented consisted of 11 mixed-breed dogs, five German Shorthair Pointers, five Labrador Retrievers, four Golden Retrievers, three German Shepherd dogs, three Bernese Mountain dogs, two Boxers, two Great Danes, and one each of Beagle, Border Collie, Miniature Dachshund, Greyhound, Leonberger, Mastiff, Newfoundland, Pembroke Welsh Corgi, Pit Bull Terrier, Russian Mountain dog, St Bernard, Australian Shepherd, and Weimaraner. There were 22 neutered males, 20 neutered females, four male entire, and two female entire dogs. Joints sampled consisted of 44 stifles, five carpi, four tarsi, and one shoulder. Two stifles were sampled twice and one stifle was sampled three times. The shortest time between repeat sampling of a joint was 10 weeks. Clinical diagnoses were cranial cruciate ligament (CrCL) rupture ($n = 13$), immune-mediated polyarthritis (IMPA) ($n = 9$), septic arthritis ($n = 4$), osteoarthritis ($n = 4$), lateral patella luxation ($n = 2$), combined CrCL rupture and IMPA ($n = 3$), meniscal tearing ($n = 2$), and one each of trauma, sarcoma, osteochondrosis, and carpal hyperextension injury. A definitive diagnosis remained open in seven dogs.

Automated TNCC and manual counts

Automated TNCC ranged from 60 to 118,100 cells/ μL . The TNCC was WRI for 32 samples, mildly to moderately elevated for 10 samples, and markedly increased for 16 samples. Mean manual counts ranged from 0.4–172.7/400 $\times$ field. There was a significant positive correlation between the mean number of cells/400 $\times$ field and the automated TNCC ($S_R = 0.85$, $P < 0.001$). This relationship was more variable at higher cell counts (Figs. 2 and 3). A table was constructed to convert mean number of cells/400 $\times$ field to an estimated TNCC/ μL (see Appendix: Supplementary material, Table S1).

When the regression equation was used to predict TNCC from the number of nucleated cells/400 $\times$ field and categorize the nucleated cell count by level, TNCC category was predicted correctly in 45 of 58 (78%) samples (Fig. 4). When TNCC category was predicted incorrectly, samples with automated TNCC WRI were overestimated (10/32; 31%), whereas samples with automated TNCC markedly increased were underestimated (2/16; 13%; Fig. 4A). In one sample with a mild to moderately increased automated TNCC, estimated TNCC was predicted to be markedly increased. Tests for overall marginal homogeneity between manual counts and automated measurement of TNCC were significant ($P < 0.05$). For samples with WRI automated counts, estimated TNCC predicted a significantly greater proportion of samples with mild to moderate increases ($P < 0.005$) when compared with automated TNCC. For samples with mild to moderately increased automated counts, the regression formula used for estimating TNCC predicted a significantly greater proportion of samples as WRI or markedly increased ($P < 0.005$) (Fig. 4B).

Intra-observer and inter-observer variation in manual counting

Assessment of intra-observer reliability showed excellent agreement among observations. Inter-observer reliability showed good agreement among observers (Table 1). Reliability of smear preparation was excellent (Table 1).

Discussion

Automated TNCC and manual cell counts showed a significant degree of correlation and variation in automated TNCC was explained to a large degree by the number of nucleated cells/400× field on direct smears counted using our method. Furthermore, when automated counts were partitioned into three clinically relevant categories, manual cell counts predicted the correct category for the majority of samples. It is advantageous to estimate TNCC and obtain a differential cell count from a single direct smear, when automated counts are not possible.

TNCC is used in both initial diagnosis and monitoring response to treatment (Johnson and Mackin, 2012). A TNCC of 3000 cells/μL is accepted as a threshold value, at the upper limit of the WRI (Barger, 2010; Johnson and Mackin, 2012; Fernandes, 2014). In the absence of an automated TNCC, an estimated count can be obtained from direct smears. One study has proposed counting cells at 100× magnification and multiplying by 100 (Barger, 2010), whereas another recommends multiplying the number of cells/400× field by 1000 (Gibson et al., 1999), suggesting that each cell represents a TNCC of 1000/μL. The latter has been cited repeatedly (MacWilliams and Friedrichs, 2003; Goldstein, 2010). Our data indicate that these relationships provide a poor estimate of true TNCC, as each counted cell represents considerably fewer cells/μL than predicted by these studies. The discrepancy in the correlative relationship between estimated and actual TNCC may be related to the region of the smear where counting is performed and smear preparation technique.

The ability to accurately estimate TNCC is important, as this value is the gold standard for classifying fluid as normal or inflamed (Barger, 2010; Fernandes, 2014). In one study, TNCC predicted from direct smears by four clinicians correlated poorly with automated counts obtained using an impedance counter (Gibson et al., 1999) and the method for estimating TNCC from slides was not described. Differential cell counts were also determined and coupled with the TNCC to categorize disease; manual counts by three of the clinicians resulted in correct classification of disease, based on automated counts and defined criteria. Although this method may have been sufficient for initial diagnosis, treatment of diseases like IMPA require longitudinal monitoring of the TNCC, as this plays an important role in treatment decisions (Johnson and Mackin, 2012). Additionally, studies utilizing changes in TNCC to evaluate drug efficacy, rely on accurate cell counts. Thus, estimation of TNCC is valuable when an automated TNCC is unavailable.

Although the estimated TNCC in our study showed good agreement with the automated TNCC, the confidence interval (CI) increased as TNCC increased. At 7–10 nucleated cells/400× field, the 95% CI spanned the threshold of 3000 cell/μL, making categorization of counts as WRI or mildly increased, less certain. The category of TNCC was not predicted correctly by the model in 22% of samples, with mis-categorization of samples WRI or mild

to moderately increased to a higher category and underestimation of samples with markedly increased TNCC. Overestimation of increased TNCC is preferable to underestimation for monitoring response to therapy, as it is better to treat inflammation for longer than necessary than to withdraw treatment prematurely. However, overestimation of a TNCC WRI may result in failure to withdraw immunosuppressive therapy when they are no longer necessary. Differential cell counts and clinical presentation should also be considered, along with TNCC, to evaluate various types of joint disease.

Intra-observer and inter-observer agreement for TNCC estimation in the present study was good to excellent, contrasting with a previous study in which marked inter-observer variation was reported (Gibson et al., 1999). TNCC estimation from smears can be difficult (Barger, 2010; Fernandes, 2014), especially when experience in cytopathology is limited. Intra-observer and inter-observer variation in the present study was minimized by following a standard operating procedure. Individuals with minimal microscope experience were able to achieve similar results to more experienced observers, by following the established protocol.

The study had several limitations. Few samples had an automated TNCC around the critical point of 3000/ μ L. All smears were prepared by highly trained staff, but exclusion criteria related to smear width and length and number of ruptured nucleated cells were required. Thin smears of synovial fluid with a feathered edge can be difficult to make (Barger, 2010; Fernandes, 2014). It is not known what effect poorly constructed smears might have on the regression analysis. None of the samples in the present study contained substantial amounts of hemorrhage; hemodilution of synovial fluid may alter its smearing properties and thus influence manual counts. This alteration may explain increased variability in estimated TNCC in samples with high TNCC, as viscosity tends to decrease with increasing inflammation (Barger, 2010; Fernandes, 2014). The study focused on canine synovial fluid samples, which might be extrapolated to other veterinary species. Although reference intervals for feline TNCC have been published and acquisition of sufficient volume for analysis is problematic (Pacchiana et al., 2004), orthopaedic disease in cats is relatively uncommon. Equine arthrocentesis, on the other hand, generally provides an adequate sample volume for automated TNCC (Mahaffey, 2002).

Conclusions

We describe a standard operating procedure for performing manual counting of nucleated cells on direct smears of canine synovial fluid for TNCC estimation. Validation of the methodology and results by other laboratories is indicated, because of inherent variability in direct smear preparation. Although the method is somewhat labor-intensive and requires meticulous smear preparation, this technique could also enable refinement of therapeutic monitoring of joint disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Atilola MAO, Lumsden JH, Rooke F. A comparison of manual and electronic counting for total nucleated cell counts on synovial fluid from canine stifle joints. *Canadian Journal of Veterinary Research*. 1986; 50:282–284. [PubMed: 3756682]
- Aulesa C, Mainar I, Prieto M, Cobos N, Galimany R. Use of the Advia 120 hematology analyzer in the differential cytologic analysis of biological fluids (cerebrospinal, peritoneal, pleural, pericardial, synovial, and others). *Laboratory Hematology*. 2003; 9:214–224. [PubMed: 14649464]
- Barger, AM. *Canine and Feline Cytology*. 2. Saunders Elsevier; St. Louis, MO, USA: 2010. Musculoskeletal system; p. 309-324.
- Ekmann A, Rigdal ML, Grondahl G. Automated counting of nucleated cells in equine synovial fluid without and with hyaluronidase pretreatment. *Veterinary Clinical Pathology*. 2010; 39:83–89. [PubMed: 20051063]
- Fernandes, PJ. Cowell and Tyler's *Diagnostic Cytology and Hematology of the Dog and Cat*. 4. Elsevier Mosby; St. Louis, MO, USA: 2014. Synovial fluid analysis; p. 195-215.
- Gibson NR, Carmichael S, Li A, Reid SWJ, Normand EH, Owen MR, Bennett D. Value of direct smears of synovial fluid in the diagnosis of canine joint disease. *Veterinary Record*. 1999; 144:463–465. [PubMed: 10358874]
- Goldstein, RE. *Textbook of Veterinary Internal Medicine*. 7. Saunders Elsevier; St. Louis, MO, USA: 2010. Swollen joints and lameness; p. 130-133.
- Johnson KC, Mackin A. Canine immune-mediated polyarthritis. Part 2: Diagnosis and treatment. *Journal of the American Animal Hospital Association*. 2012; 48:71–82. [PubMed: 22267168]
- Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977; 33:159–174. [PubMed: 843571]
- MacWilliams PS, Friedrichs KR. Laboratory evaluation and interpretation of synovial fluid. *Veterinary Clinics of North America: Small Animal Practice*. 2003; 33:153–178. [PubMed: 12512381]
- Mahaffey, EA. *Diagnostic Cytology and Hematology of the Horse*. 2. Mosby; St. Louis, MO, USA: 2002. Synovial Fluid; p. 163
- Pacchiana PD, Gilley RS, Wallace LJ, Hayden DW, Feeney DA, Jessen CR, Aird B. Absolute and relative cell counts for synovial fluid from clinically normal shoulder and stifle joints in cats. *Journal of the American Veterinary Medical Association*. 2004; 225:1866–1870. [PubMed: 15643835]
- Webb, JL.; Latimer, KS. Duncan & Prasse's *Veterinary Laboratory Medicine: Clinical Pathology*. 5. Wiley-Blackwell; Ames, IA, USA: 2011. Leucocytes; p. 58

Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/XXX>

Fig. 1.

Definitions of the microscope fields for manual counting of nucleated cells on smears of synovial fluid. (A) The start point and corresponding mark. (B) The end point, midpoint, and first row of counting fields with corresponding marks. (C) Serpentine pattern of counting. If there was a distinct circular area where the drop of fluid was placed on the slide, the start point was where the smear began to spread width-wise from the drop; if the smear separated from the drop at different points on either side of the drop, the midpoint was chosen (red asterisk and lines).

Fig. 2.

Passing Bablok regression of the relationship between manual mean nucleated cell count/400× field and the automated total nucleated cell count (TNCC) ($n = 58$ samples). The relationship is positive and more variable at higher counts. $S_R = 0.852$, $P < 0.001$, $Y = -0.36 + 0.45 * X$. The identity line (equivalent values, red dashed line) and the 95% confidence bounds (blue dashed lines) are also plotted.

Fig. 3.

(A) Scatter plot of the relationship between estimated total nucleated cell count (TNCC) in canine synovial and the automated TNCC ($n = 58$ samples). The automated TNCC variable is explained to a large degree by counting numbers of nucleated cells/400 $\times$ field from direct smears using the method described. (B) Bland-Altman plot of TNCC difference (Estimated TNCC – Automated TNCC) versus TNCC average. At higher TNCC values, the manual counting method tends to underestimate TNCC, whereas at lower TNCC values, the manual counting method tends to overestimate TNCC.

Fig. 4.

(A) Relationship between estimated total nucleated cell counts (TNCC) from manual counting of direct smears and automated TNCC categorized as within the reference interval (WRI; <3000 cells/ μL), mild to moderately (mod) increased (3000 – $20,000$ cells/ μL), or markedly increased ($>20,000$ cells/ μL). Frequency indicates the number of synovial fluid samples (total $n = 58$ samples). (B) Significance of categories by manual and automated counting.

Table 1

Intra-observer and inter-observer reliability for manual counting of cells on direct smears of synovial fluid.

	Intra-class correlation	95% confidence interval
Intra-observer reliability	0.998	0.995–0.999
Inter-observer reliability for experienced observers	0.753	0.390–0.920
Inter-observer reliability for inexperienced observers	0.719	0.365–0.904
Inter-observer reliability for all observers	0.792	0.567–0.927
Reliability of smear preparation	0.965	0.886–0.996

Note: All correlations were significant at $P < 0.001$.

Inter-observer reliability for experienced observers compared counts performed by a graduate veterinarian with experience in a clinical pathology laboratory setting (AD) with a board-certified clinical pathologist and a clinical pathology resident. Inter-observer reliability for inexperienced observers compared counts performed by a graduate veterinarian with experience in a clinical pathology laboratory setting (AD) with a laboratory technician with limited experience in microscopy and a student research assistant with no experience evaluating or interpreting canine cytological samples. For reliability of smear preparation, a single observer (AD) performed all the counts.