Flow cytometry for feline lymphoma: a retrospective study regarding pre-analytical factors possibly affecting the quality of samples

| Abstract: | Objective Flow cytometry (FC) is becoming increasingly popular among veterinary oncologists for the diagnosis of lymphoma or leukemia. It is accurate, fast, and minimally invasive. Several studies on FC have been carried out in canine oncology and applied with great results, whereas there is limited knowledge and use of this technique in feline patients. This is mainly due to the high prevalence of intra-abdominal lymphomas in this species and the associated discomfort in the diagnostic procedures needed to collect the sample. The purpose of the present study is to investigate whether any pre-analytical factor might affect the quality of suspected feline lymphoma samples for FC analysis.
Methods 97 consecutive samples of suspected feline lymphoma were retrospectively selected from the authors' institution FC database. The referring veterinarians were contacted and interviewed about several different variables, including signalment, appearance of the lesion, features of the sampling procedure and the experience of veterinarians performing the sampling. Statistical analyses were performed to assess the possible influence of these variables on the cellularity of the samples and the likelihood of it being finally processed for FC.
Results Sample cellularity is a major factor in the likelihood of the sample being processed. Moreover, sample cellularity was significantly influenced by the needle size, with 21G needles providing the highest cellularity. Notably, the sample cellularity and the likelihood of being processed did not vary between peripheral and intra-abdominal lesions. Approximately half of the cats required pharmacological restraint. Side effects were reported in one case only (transient swelling after peripheral lymph node sampling).
Conclusion and relevance FC can be safely applied to cases of suspected feline lymphomas, including intra-abdominal lesions. 21G needle should be preferred for sampling. This study provides the basis for the increased use of this minimally invasive, fast and cost-effective technique in feline medicine. |

1 **Flow cytometry for feline lymphoma: a retrospective study <mark>regarding</mark> pre-**

2 **analytical factors possibly affecting the quality of samples**

3 Martini V[1], Bernardi S[1], Marelli P[1], Cozzi M[1], Comazzi S[1].

4 [1] Department of Veterinary Medicine, University of Milan, Milan, Italy

5

6 **Corresponding author:**

7 VALERIA MARTINI, DVM, PhD

8 Department of Veterinary Medicine

9 University of Milan

10 Via Celoria 10 20133 Milan, Italy

11 Phone: +39 0250318153

12 valeria.martini@unimi.it

13

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Abstract

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Results Sample cellularity is a major factor in the likelihood of the sample being processed. Moreover, sample cellularity was significantly influenced by the needle size,

36   with 21G needles providing the highest cellularity. Notably, the sample cellularity and

37   the likelihood of being processed did not vary between peripheral and intra-abdominal

38   lesions. Approximately half of the cats required pharmacological restraint. Side effects

39   were reported in one case only (transient swelling after peripheral lymph node

40   sampling).

41   **Conclusion and relevance** FC can be safely applied to cases of suspected feline

42   lymphomas, including intra-abdominal lesions. 21G needle should be preferred for

43   sampling. This study provides the basis for the increased use of this minimally invasive,

44   fast and cost-effective technique in feline medicine.

45

Introduction

Lymphoproliferative malignancies are fairly common in dogs and cats.[1] In the canine

species, the diagnosis of lymphoma and leukaemia is nowadays easy, fast and

minimally invasive thanks to useful tools like flow cytometry (FC).[2] FC is widely used

in canine oncology, not only for the determination of neoplastic cell lineage, but also

because of the increasing number of available leucocyte markers, which strengthens the

chance to recognize different lymphoma subtypes.[3,4] Moreover, in dogs FC allows to

assess the stage of the tumour or the minimal residual disease (MRD) after treatment.[5,6]

In cats, the prevalence of lymphoma is believed to be high: in the 1970s and 1980s one

third of feline neoplasms was estimated to be of hematopoietic origin, either lymphoid

or myeloid, in association with a high prevalence of feline leukaemia virus (FeLV)

infections. Since late 1980s the situation has changed: the prevalence of FeLV+ cats and

associated forms of hematopoietic tumours decreased thanks to efficient diagnostic

tests, vaccination and infected cats isolation, whereas the number of not-FeLV-related

forms of lymphoid tumours increased (mostly alimentary and cutaneous forms).[7]

Cytology of suspected feline lymphomas is often heterogeneous and thus generally not

conclusive for neoplasia. Histopathology and immunohistochemistry remain the gold

standard for the diagnosis and immunophenotyping of feline lymphoma,[8] but these

techniques are invasive, as they require a biopsy specimen, and time-expensive, as some

days are needed for results.

66   FC could overcome these limits, although only a single study has been published so far

67   on this topic.[9] In spite of the high prevalence of lymphoma in cats, the application of FC

68   for the diagnosis and characterization of this tumour in the feline species is still limited

69   and considered challenging for several possible reasons.[10] Firstly, because of the high

70   prevalence of intra-abdominal forms, which are anecdotally reported to yield poor

71   quality samples because of sampling difficulties. Secondly, the availability of species-

72   specific monoclonal antibodies (mAbs) binding to feline leukocyte differentiation

73   antigens (LDA) is restricted for FC application and only a few studies have been

74   conducted for evaluation of cross reactivity with mAbs of other species.[11,12]

75   The general thought concerning the difficulty of obtaining good quality samples from

76   abdominal lesions in cats is unsubstantiated, and has never been documented (nor

77   contradicted) in the scientific literature. Going the long way round, to the authors'

78   knowledge, there are no published studies concerning the quality of samples for FC

79   analysis of feline lymphomas.

80   The aim of the present study was to evaluate if and which specific pre-analytical factor

81   could affect the quality of feline lymphoma samples for FC and, as a consequence, the

82   likelihood of being processed. Factors taken into account concerned both sampling and

83   processing procedures. This might be an important assessment in order to provide useful

84   indications for a good quality sampling for FC in cats with suspected lymphomas.

85   The use of FC may improve the diagnosis and classification of feline lymphomas,

86   raising it to the levels achieved in the dog. However, the assessment of the diagnostic

87   accuracy of FC for feline lymphomas does not fall within the aims of the present study,

88   as it has already been described in the literature.[9]

89

90   **Materials and methods**

91   The database of the FC service of the authors' Institution from January 2009 to

92   February 2016 was interrogated and feline cases were extracted. Inclusion criteria were:

93   1) cases sent to the laboratory for suspected lymphoma; 2) request for FC

94   immunophenotyping of the primary lesion, including effusions. Cases were excluded

95   from the study if only peripheral blood and/or bone marrow samples had been sent to

96   the laboratory.

97   For each case, data concerning the subject, the lesion, the sampling technique, the

98   ancillary tests performed as well as data concerning the clinician who collected the

99   sample were requested from the referring veterinarian, if not provided at the time of

100   sample submission (Table 1). Since no objective parameters exist to assess and quantify

101   the veterinarians' clinical experience, this was evaluated with two parameters set for

102   this study. These latter were both the timespan between the veterinarians license

103   acquisition and the sample collection (years of expertize) and the presence of any post-

104   graduate specialization, irrespective of the type of specialization and its specific field of

105   application (whether clinical or not). If samples from multiple sites were available for a

106   single animal, the data were recorded for each sample independently. Additional data

107   were retrieved from the FC database (Table 1).

108   All FC data were reviewed by a single operator (VM) who was aware of the cytological

109   diagnosis (when available) but was blinded to the previous FC report and to all other

110   tests performed on the lesion.

111   *Flow cytometry*

112   FC was performed on tissue aspirates collected in a liquid medium (either saline

113   solution or RPMI 1640) or on effusions collected in EDTA-tubes with an adaptation of

114   a previously published procedure.[9]

115   Prior to labelling, all samples were counted via an automated haematology analyser

116   (Sysmex XT-2000iV, Sysmex, Kobe, Japan) to assess cellularity. Also, a visual

117   inspection of the sample was made by the operator, to assess both the total volume of

118   the sample and the presence of artifacts such as gross hemocontamination, clots,

119   necrotic material, or any other abnormality. Based on all these parameters, the operator

120   dealing with each sample decided whether to admit it to FC or not, based on his/her

121   subjective opinion and experience.

122   The antibody panel varied among samples: indeed, for samples processed between

123   January 2009 and December 2010, a single-color approach was used with the inclusion

124   of a FITC-conjugated secondary antibody (rabbit anti-mouse IgG-FITC, polyclonal,

125   Serotec), whereas a multi-colour approach was used for samples processed between

126   January 2011 and February 2016 (CD5-FITC/CD21-PE/CD18-AlexaFluor647; CD4-

127   FITC/CD8-PE/CD18-AlexaFluor647). Antibody clones and source are listed in Table 2.

128   All samples were acquired with a FACScalibur flow cytometer (Becton Dickinson, San

129   Josè, CA, USA) and analysed with a specific software (CellQuest, Becton Dickinson).

130   *Statistical analysis*

131   Statistical analyses were performed to assess whether the cellularity of FC samples and

132   the likelihood of samples to be finally processed for FC were influenced by any pre-

133   analytical variable (Table 1).

134   To this aim, continuous variables were arbitrarily categorized as follows: age of cat (≤1

135   or >1 year); years of experience of the vet (i.e. timespan between veterinarian license

136   acquisition and sample collection, <15 or ≥15 years). The lesions were classified into

137   five groups based on their site: peripheral lymph nodes (pLN), abdominal lymph nodes

138   or masses (aLN), thoracic lymph nodes or masses (tLN), effusions (grouped altogether

139   irrespective of their primary location, either thoracic or abdominal), and any other site.

140   Contingency tables were prepared for each of the investigated variables, and the

141   Pearson χ^2 test was performed to assess their possible association with the likelihood of

142   samples to be processed for FC.

143   Shapiro-Wilk test was performed to assess whether the FC samples cellularity was

144   normally distributed. Then, Student t-test, Mann-Whitney test, ANOVA or Kruskal-

145   Wallis test were performed to compare the mean sample cellularity among different

146   categories, based on the data distribution (normal or not) and the number of groups (2 or

147   more). The Kruskal-Wallis test was also performed to compare the mean cellularity

148   among samples with different FC diagnosis (conclusive for lymphoma, negative for

149   lymphoma, not conclusive): this analysis was restricted to samples finally processed for

150   FC. When significant results were obtained, appropriate post-hoc tests were performed

151   based on data distribution and homoscedasticity assessment (Mann-Whitney,

152   Bonferroni or Dunnett test).

153   Initially, all samples were included in the analyses. Thereafter, samples were

154   investigated according to the five lesion site groups.

155   All analyses were performed with SPSS v20.0 for Windows. Significance was set at

156   P≤0.05 for all tests.

157

158   **Results**

159   105 consecutive suspect lymphoma samples were retrospectively extracted from the FC

160   service's database of DIVETLAB (Department of Veterinary Medicine, University of

161   Milan). Eight were then excluded from the study for different causes: three were

162   delivered to the laboratory 48 hours after sampling, three were sampled after the

163   administration of chemotherapy. Lastly, in two cases the sampling was repeated twice

164   because the first one had a low cellular concentration and had not been processed: in

165   these two cases, only the first (poorly cellular) sample was included in the study. Thus,

166   97 samples were finally included in the present study, from 86 different feline patients:

167   73 (75.3%) out of these samples were analysed for FC, while the remaining 24 (24.7%)

168   were discarded by the operator. Thirty-one (32%) samples were collected before 2011

169   and were analysed with a single-color approach while 66 (68%) samples were collected

170   subsequently and analysed with a multi-colour approach. The proportion of samples

171   finally processed for FC did not vary with year of sampling, nor with the FC approach

172   used.

173   Total nucleated cell count (TNCC) was reported for 91 samples, with a mean of $12.96 \pm$

174   21.19×10^3 cells/μL (median: 3.11×10^3 cells/μL; minimum-maximum: 0.01-$89.88 \times$

175   10^3 cells/μL). In particular, it was significantly higher in samples eventually processed

176   for FC (mean: $14.78 \pm 22.12 \times 10^3$ cells/μL; median: 4.09×10^3 cells/μL; minimum-

177   maximum: 0.16-89.88×10^3 cells/μL) than in discarded samples (mean $7.26 \pm 17.20 \times$

178   10^3 cells/μL; median: 0.54×10^3 cells/μL; minimum-maximum: 0.01-58.02×10^3

179   cells/μL; P=0.000). None of the remaining variables significantly influenced the

180   likelihood of samples of being finally processed for FC.

181   In turn, TNCC was significantly influenced by the size of the needle and by the

182   presence of post-graduate specialization of the veterinarian performing the sampling.

183   The 21 G needle gave the most cellular samples, with a statistically significant

184   difference from the other needles (P=0.045). Size of the needles with relative average

185   cellular concentration are listed in Table 3. Of 37 samples for which this information

186   was available, 33 (89.2%) were collected by veterinarians with post-graduate

187   qualifications: veterinarians with post graduate qualifications collected samples with an

188   average cellular concentration of 9.42 ± 19.86 x10^3 cells/μL (median 1.71 x10^3 cells/μL;

189   minimum-maximum 0.01-87.54 x10^3 cells/μL), whereas other first opinion veterinarians

190   collected samples with an average cellular concentration of 33.16 ± 29.5 x10^3 cells/μL

191   (median 34.01 x10^3 cells/μL; minimum-maximum 5.35-59.26 x10^3 cells/μL). The

192   difference was statistically significant (p=0.027).

193   None of the other variables (including lesion site and size) gave significant results. Raw

194   results are listed below.

195   Breed was known for 75 cats: 64 (85.3%) domestic shorthair (DSH), 4 (5.3%) Maine

196   Coon, 2 (2.7%) British shorthair, 2 (2.7%) Chartreux, 2 (2.7%) Persian and 1 (1.3%)

197   Norwegian Forest. Sex was known for 81 cats: 18 (22.2%) were intact females, 21

198   (25.9%) were neutered females, 9 (11.1%) were intact males and 33 (40.7%) were

199   neutered males. Age was known for 85 cats, who aged from 5 months to 16 years, with

200   a median age of 8 years. FIV/FeLV status was known only for 16 patients: 7 (43.8%)

201   were negative for both, 7 (43.8%) were FeLV+ and 2 (12.5%) were FIV+.

202   The site of the lesion was known for all 97 samples: 24 (24.7%) pLN, 21 (21.6%) aLN,

203   21 (21.6%) tLN, 17 (17.5%) effusions and 14 (14.4%) other sites, including skin,

204   kidney, spleen, liver and urine. Lesion's size was known for 49 samples: 13 (26.5%)

205   were beneath 2 cm, 22 (44.9%) were between 2 and 5 cm and 14 (28.6%) measured

206   more than 5 cm.

207   Concerning cytology, 67 samples were received with a cytological report: of these, 30

208   (44.8%) were conclusive for lymphoma, for 16 (23.9%) lymphoma was suspected with

209   different confidence levels (diagnostic but not conclusive), for 9 (13.4%) lymphoma

210   was excluded, 1 (1.5%) was diagnostic for thymoma (for a total of 10 lymphoma-

211   negative samples) and 11 (16.4%) were considered non-diagnostic because of poor

212   cellular concentration, high hemodilution or poor quality of the preparation.

213   The method of sampling for FC was known for 66 samples: 16 (24.2%) were made by

214   blind aspiration, 41 (62.1%) ultrasound-guided, 7 (10.6%) computed tomography-

215   guided, 1 (1.5%) was obtained by surgical access and 1 (1.5%) by urethral

216   catheterization. Patient waking condition was known for 53 samples: 24 (45.3%) were

217   awake, 17 (32.1%) needed mild sedation, 12 (22.6%) needed general anaesthesia.

218

219   Of the 44 patients for which this information was available, side effects of sampling

220   were reported in one case only: this cat showed a transient mild swelling in the

221   sampling region (submandibular lymph node).

222   Transport medium was known for 58 aspirates: 6 (10.3%) were collected in saline

223   solution and 52 (89.7%) were collected in culture medium (RPMI or DMEM). All

224   effusions were collected in EDTA tubes.

225   Concerning the experience of the clinician performing the sampling, the timespan

226   between graduation and sample collection was < 15 years for 25 (46.3%) samples and >

227   15 years for 29 (53.7%) samples.

228

229   As a second step, the statistical analyses were performed including samples according to

230   the five different lesion sites (pLN, aLN, tLN, effusions, and other sites). Results are

231   reported in the supplementary materials (Tables S1 to S10). Significant differences were

232   noted only within the tLN group: clinicians without postgraduate qualifications

233   collected samples with a higher cellularity (P=0.036).

234

235   Finally, cellular concentration was evaluated according to the FC diagnosis. TNCC was

236   not recorded for 4 samples. The remaining 69 processed samples were divided in three

237   categories: positive for lymphoma, negative for lymphoma and non-diagnostic. TNCC

238   significantly varied among the three groups (P=0.022; Table 4): in particular, non-

239   diagnostic samples were less cellular than lymphoma and non-lymphoma samples

240   (P=0.009 and P=0.040, respectively). The difference in TNCC according to FC

241   diagnosis remained significant also within the pLNs and effusions groups (P=0.029 for

242   both): the significant difference was between lymphoma and non-diagnostic samples for

243   both groups (P=0.016 for pLNs and P=0.036 for effusions). TNCC values are shown in

244   Table 5 and 6.

245

246   **Discussion**

247   FC is widely used in human medicine and its use in veterinary medicine has been

248   increasing in the last years, especially for canine lymphoproliferative diseases. In the

249   canine species, this diagnostic tool turned out to be very helpful for a rapid and non-

250   invasive lymphoma diagnosis.[2] Moreover, some studies have been published in the last

251   years about the prognostic value of the flow cytometric immunophenotype in this

252   species.[5,13,14]

253   Nevertheless, in the feline species, FC is not commonly used; to the authors' knowledge,

254   it was never described in the last decade until last year, when Guzera *et al* published the

255   first scientific study about the application of FC in the diagnosis of feline lymphoma,

256   highlighting the diagnostic accuracy of this technique.[9]

257   Being most of feline lymphomas localized in the intra-abdominal region, reaching the

258   primary lesion with a needle might be uncomfortable for the clinician, and mild or

259   general anaesthesia may be necessary. Based on this general thought, FC is usually not

260   included in the diagnostic workup for suspected feline lymphomas. The lack of

261   confidence in this technique is confirmed by the fact that feline samples represent only

262   about 2% of the samples included in the authors' FC database in the last seven years

263   (2009-2016).

264   The study published by Guzera et al[9] and the present study somehow deny this common

265   belief, because a high number of samples in both studies were likely to be processed

266   and to be diagnostic. In the present study, 75% of samples were finally processed for

267   FC; of these, only 20% were non-diagnostic and they had a lower cellular concentration

268   compared to the diagnostic samples. In our laboratory, samples are usually admitted to

269   be processed for FC only if suitable to be analysed with the whole antibody panel,

270   irrespective of the FC approach used (single- VS multi-colour). The percentage of

271   processed samples in the study by Guzera and colleagues[9] was slightly higher, but only

272   a limited antibody panel was applied to a subset of samples, which may explain this

273   discrepancy between the two studies. Summarizing the results of the two studies, we

274   could state that 75-85% of feline samples is suitable for FC analysis. Samples with a

275   low cellular concentration could still be investigated through a more limited panel of

276   antibodies, although they are less likely to be of diagnostic usefulness.

277   Based on our results, cellular concentration is a key-point in the discrimination between

278   samples suitable or unsuitable for FC. Still, some poorly cellular samples were admitted

279   to processing and, *vice versa*, some highly cellular samples were not. The choice

280   whether to process or not the samples was left to the operator dealing with the sample,

281   and was likely based also on other features, resulting from the visual inspection of the

282   sample, together with the TNCC. Unfortunately, these data were not reported in the FC

283   records and their influence on TNCC and on the likelihood of samples of being

284   processed could not be evaluated in the present study. Still, gross inspection of the

285   specimen is recommended, before admission to processing for FC.

286   In our case series, similar numbers of pLN, aLN and tLN were present, in spite of the

287   higher prevalence of alimentary lymphoma reported in cats.[15,16] One possible

288   explanation for this discrepancy is the presence of many non-lymphoma cases. Another

289   possible reason is that clinicians prefer not to sample hardly reachable lesions such as

290   gastro-intestinal lesions, spleen, liver or aLN, due to a supposed poor quality of the

291   sample. Still, our data support the application of FC even for intra-abdominal lesions, as

292   these had the same likelihood of being finally processed for FC than the aspirates taken

293   from peripheral lymph nodes (which are expected to be more comfortably reached).

294   Although sedation or anaesthesia of the cat may be of aid to the clinician during

295   specimen collection, these did not ensure to catch higher quality samples. Thus, they are

296   not essential and the choice whether to use them or not should only be based on the

297   cat's character. The possible occurrence of side effects might worry the operator as

298   well. However, for the cases included in the present study, no side effects have been

299   reported following intra-thoracic/abdominal fine needle aspiration (FNA), but just one

300   patient had a transient swelling after FNA of a pLN.

301   Among all the factors we evaluated, only two of them affected significantly the cellular

302   concentration of samples: the size of the needle and the post-graduation qualification of

303   the clinician.

304   The results show that 21G needle, a medium size needle, is related with a higher cellular

305   concentration of the sample. On one hand, smaller needles could damage the cells, that

306   are more fragile for their neoplastic origin, and necrotic or clotted material or

307   connective tissue could plug the needle. On the other hand, larger needles could be more

308   traumatic on the tissue, producing bleeding and thus contamination of the sample with

309   too much blood and other surrounding tissues (necrosis, connective, fat). Also, the small

310   number of samples collected with different needle sizes may have influenced the

311   statistical results. Still, following these results, the advice is to use 21G needle to have

312   good quality samples.

313   The fact that less cellular samples came from theoretically more qualified veterinarians

314   is surprising: the most probable explanation for this result is a statistical artefact due to

315   few samples coming from operators without post-graduate qualification (4). Most of

316   these clinicians regularly send canine samples to our FC service, so they have a steady

317   practical experience in sampling for this purpose. Otherwise, new inexperienced

318   operators may have asked to the FC service's staff for instructions about sampling,

319   transport, medium and sample storage prior to sampling. However, this result is of

320   questionable value and should be better addressed by future studies.

321   One of the most important result from the present study is that the likelihood of

322   processing the sample and the cellular concentration are not affected by size and site of

323   the lesion, unlike what has been thought until now. This makes FC appropriate for

324   application in the feline species, even if lesions are intra-abdominal or thoracic.

325   In our case series, less than a half of cytological preparations was conclusive for

326   lymphoma: despite this poor diagnostic value of cytology alone for the diagnosis of

327   feline lymphomas, this test remains a mandatory first step in the diagnostic workup. In

328   addition, a subset of samples in the present study were sent to our laboratory with a

329   suspect of lymphoma even in face of a negative cytological report. Although the

330   referring veterinarians have provided no clear explanation for this phenomenon, it may

331   be considered a proof of the scarce confidence of clinicians toward negative cytological

332   reports, when a strong suspicion of neoplasia is present based on clinical signs and

333   imaging. The diagnostic performances of cytology alone and cytology plus FC is still to

334   be elucidated in a clinical setup.

335 This is only the second paper published concerning FC as a diagnostic tool for feline

336 lymphoma patients. Clinicians are only slightly familiar with this technique and there is

337 the need to enhance their confidence, based on its promising large spread in the human

338 and canine species. Thus, we support the contemporary sampling for FC and

339 histopathology/immunohistochemistry in cases of suspected feline lymphomas: this

340 would provide a rapid report (within 24 hours) from FC and a subsequent confirmation

341 and more detailed classification from histology/immunohistochemistry.

342 The retrospective formulation of the present study is its main limitation: information

343 collected were often incomplete and there was no standard sampling procedure.

344 Confirmation will be necessary in the future, through a prospective sample collection

345 and a complete submission of the case. Another main limitation of the present study is

346 the lack of a confirmatory test, as histology was available only for few cases (data not

347 shown) and PARR for none. This prevented us from assessing the diagnostic accuracy

348 of FC for feline lymphomas; anyway, Guzera et al. already evaluated it in their study,

349 though on a narrow sample.[9]

350

351 **Conclusions**

352 The results of the present study show how FC can be used for immunophenotyping in

353 feline lymphomas, regardless of the site and the size of the lesion sampled. The use of

354   21G needles may enhance the probability to catch highly cellular samples. This is a

355   pilot study aimed at making FC more widely known in the feline medicine world, and

356   future studies are necessary to make this tool as useful as it currently is in dogs, from

357   both a diagnostic and a prognostic point of view.

358

359   **Supplementary material**

360   Results obtained from the analyses within lesion-site groups are available as

361   Supplementary material.

362

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370

371   **References**

372   1. Vail DM, Pinkerton ME and Young KM. Hematopoietic tumors. In: SJ

373   Withrow, DM Vail and RL Page (eds) *Withrow and MacEwen's Small Animal*

374   *Clinical Oncology.* St. Louis, MO: Elsevier Health Sciences, 2013, pp. 608-637

375   2. Comazzi S and Gelain ME. Use of flow cytometric immunophenotyping to

376   refine the cytological diagnosis of canine lymphoma. *Vet J* 2011; 188(2): 149-

377   155

378   3. Seelig DM, Avery P, Webb T et al. Canine T-zone lymphoma: Unique

379   immunophenotypic features, outcome, and population characteristics. *J Vet*

380   *Intern Med* 2014; v28: 878–886

381   4. Martini V, Poggi A, Riondato F et al. Flow cytometric detection of phenotypic

382   aberrancies in canine small clear cell lymphoma. *Vet Comp Oncol* 2015; 13:

383   281–287

384   5. Marconato L, Martini V, Aresu L et al. Assessment of bone marrow infiltration

385   diagnosed by flow cytometry in canine large B cell lymphoma: Prognostic

386   significance and proposal of a cut-off value. *Vet J* 2013; 197: 776–781

387   6. Aresu L, Aricò A, Ferraresso S, et al. Minimal residual disease detection by flow

388   cytometry and PARR in lymph node, peripheral blood and bone marrow,

389   following treatment of dogs with diffuse large B-cell lymphoma. *Vet J* 2014;

390   200: 318–324

391   7. Louwerens M, London CA, Pedersen NC et al. Feline lymphoma in the post-

392   feline leukaemia virus era. *J Vet Intern Med* 2005; 19(3): 329-335

393   8. Moore PF, Rodriguez-Bertos A and Kass PH. Feline gastrointestinal lymphoma:

394   mucosal architecture, immunophenotype, and molecular clonality. *Vet pathol*

395   2012; 49(4): 658-668

396   9. Guzera M, Cian F, Leo C et al. The use of flow cytometry for

397   immunophenotyping lymphoproliferative disorders in cats: a retrospective study

398   of 19 cases. *Vet Comp Oncol* 2016; 14(1): 40-51

399   10. Burkhard MJ, Bienzle D. Making sense of lymphoma diagnostics in small

400   animal patients. *Vet clin north am small anim pract* 2013; 43(6): 1331-47

401   11. Brodersen R, Bijlsma F, Gori K et al. Analysis of the immunological cross

402   reactivities of 213 well characterized monoclonal antibodies with specificities

403   against various leucocyte surface antigens of human and 11 animal species. *Vet*

404   *Immunol Immunopathol* 1998; 64(1): 1-13

405   12. Meister RK, Taglinger K, Haverson K et al. Progress in the discovery and

406   definition of monoclonal antibodies for use in feline research. *Vet Immunol*

407   *Immunopathol* 2007; 119(1-2): 38-46

408   13. Comazzi S, Gelain ME, Martini V et al. Immunophenotype predicts survival

409   time in dogs with chronic lymphocytic leukaemia. *J Vet Intern Med* 2011; 25:

410   100–106

411   14. Rao S, Lana S, Eickhoff J et al. Class II major histocompatibility complex

412   expression and cell size independently predict survival in canine B-cell

413   lymphoma. *J Vet Intern Med* 2011; 25: 1097–1105

414   15. Richter K. Feline gastrointestinal lymphoma. *Vet clin north am small anim*

415   *pract.* 2003; 33(5): 1083-1098

416   16. Patterson-Kane JC, Kugler BP, Francis K. The possible prognostic significance

417   of immunophenotype in feline alimentary lymphoma: a pilot study. *J comp*

418   *pathol* 2004; 130(2-3): 220-222

419

420   **Table 1**: Pre-analytical data asked to the referring veterinarians or reported in the

421   laboratory record for 97 samples of suspected feline lymphoma, sent to the laboratory

422   for flow cytometric immunophenotyping.

Feature group	Specific variables
Animal	*Breed*
	Sex (male, neutered male, female, spayed female)
	Age (years)
	FIV/FeLV status (positive or negative)
	Presenting complaint
	Clinical findings
Sampling procedure	*Sample catching* (blind aspiration, ultrasound-guided, computed tomography-guided, surgical approach, any other)
	*Pharmacological restraint (*none, sedation, general anaesthesia)
	Sampling technique (fine needle capillary biopsy, fine needle aspiration, any other)
	Needle size (G)
	Occurrence of side effects (yes or not)
Lesion	*Site* (peripheral lymph node, thoracic mass, intra-abdominal mass,

	effusion, any other)
	Size (≤2 cm, between 2 and 5 cm, ≥5 cm)
	Cytological diagnosis
	Histopathological diagnosis
	Any other test performed
Clinician who collected the sample	*Timespan between degree in Veterinary Medicine and sample collection* (years)
	Post-degree specialization, including European/American College, master, PhD (yes or not)
Flow cytometry	*Year of analysis*
	Tube of sample collection (culture medium, saline solution, EDTA, any other)
	Cell concentration (x10^3/μl)
	Sample processed (yes or not)
	Flow cytometric approach (single-color or multi-colour)

423   Italics: variable included in the statistical analyses

424

425   **Table 2** Antibodies used for flow cytometric analysis of suspected feline lymphoma

426   samples

antibody	specificity	clone	Source
CD5	T cells	FE1.1B11	Lab/UCDavis, Davis, CA
CD4	T helper cells	vpg39	Serotec, Oxford, UK
CD8	T cytotoxic cells	FE1.10E9	Serotec
CD21-PE	B cells	CA2.1D6	Serotec
CD5-FITC	T cells	f43	SouthernBiotech, Birmingham, AL, USA
CD4-FITC	T helper cells	3-4F4	SouthernBiotech
CD8-PE	T cytotoxic cells	fCD8	SouthernBiotech
CD18-AlexaFluor647	All leukocytes	CA1.4E9	Serotec

427

428

429   **Table 3** cellular concentration of 52 samples of suspected feline lymphoma sent to the

430   laboratory for flow cytometric immunophenotyping, according to the size of the needle

431   used for sampling. The mean cellular concentration significantly varied with needle size

432   (P=0.045). [a,b] significant difference at post-hoc analysis.

Needle size (G) [number of samples]	Cellularity (x 10^3 cells / μl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
18 [6]	12.67 ± 22.92	3.7	0.03	59.26
20 [1]	21.03			
21 [4][a,b]	49.61 ± 36.72	51.90	4.75	89.88
22 [30][a]	9.49 ± 20.61	2.00	0.01	87.54
23 [8][b]	5.05 ± 8.32	1.83	0.63	21.99
25 [2]	20.19 ± 0.02	20.19	20.17	20.20
27 [1]	19.14			

433

434

435   **Table 4** cellular concentration of 69 samples of suspected feline lymphoma sent to the

436   laboratory for flow cytometric immunophenotyping, according to the flow cytometric

437   diagnosis. The mean cellular concentration significantly varied among the three

438   diagnostic groups (P=0.022). [a,b] significant difference at post-hoc analysis

Diagnosis [number of samples]	Cellularity (x 10^3 cells / μl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [29][a]	23.45 ± 29.6	10.00	1.10	89.88
Non-Lymphoma [25][b]	10.73 ± 12.6	4.75	0.63	43.59
Non-Diagnostic [15][a,b]	4.76 ± 7.5	2.48	0.16	26.89

439

440

441   **Table 5** cellular concentration of 21 feline peripheral lymph node aspirates sent to the

442   laboratory for flow cytometric immunophenotyping for suspected lymphoma, according

443   to the flow cytometric diagnosis. The mean cellular concentration significantly varied

444   among the three diagnostic groups (P=0.029). [a] significant difference at post-hoc

445   analysis

Diagnosis [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [5][a]	45.96 ± 38.05	21.99	13.65	87.54
Non-Lymphoma [11]	13.32 ± 13.77	7.3	1.68	43.59
Non-Diagnostic [5][a]	4.78 ± 7.52	2.61	0.16	18.02

446

447

448   **Table 6** cellular concentration of 11 feline effusions sent to the laboratory for flow

449   cytometric immunophenotyping for suspected lymphoma, according to the flow

450   cytometric diagnosis. The mean cellular concentration significantly varied among the

451   three diagnostic groups (P=0.029). [a] significant difference at post-hoc analysis

452

Diagnosis [number of samples]	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lymphoma [5][a]	57.56 ± 26.24	66.04	21.03	89.88
Non-Lymphoma [3]	12.50 ± 14.86	4.75	3.11	29.63
Non-Diagnostic [3][a]	2.55 ± 2.30	2.34	0.36	4.94

453

454

Table S1 24 peripheral lymph nodes samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	5	2
2-5 cm	5	1
Sample catching		
Blind aspiration	12	3
ultrasound-guided	1	0
Pharmacological restraint		
None	8	2
Mild sedation	1	1
Needle size		
18 G	2	0
22 G	6	3
23 G	1	0
25 G	1	0
27 G	1	0
Sampling technique		
Fine needle aspiration	8	3
Fine needle capillary biopsy	7	0
Transport tube		
Saline solution	2	1
Culture medium	11	2
Years since graduation		
<15	9	1
≥15	5	1
Post-degree qualifications		
No	0	0
Yes	10	2

Table S2 cellular concentration of 24 peripheral lymph nodes samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / μl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	11.36±16.30	1.68	0.01	43.59
2-5 cm	20.47±33.50	5.71	2.29	87.54
Sample catching				
Blind aspiration	21.20±29.35	8.81	0.01	87.54
ultrasound-guided	0.17			
Pharmacological restraint				
None	21.27±26.87	16.40	0.22	87.54
Mild sedation	3.20±4.51	3.20	0.01	6.39
Needle size				
18 G	4.66±2.45	4.66	2.92	6.39
22 G	11.75±28.55	2.29	0.01	87.54
23 G	21.99			
25 G	20.17			
27 G	19.14			
Sampling technique				
Fine needle aspiration	13.95±26.09	2.42	0.01	87.54
Fine needle capillary biopsy	13.63±14.82	8.81	0.17	43.59
Transport tube				
Saline solution	2.95±3.16	2.29	0.17	6.39
Culture medium	23.28±31.14	13.65	0.01	87.54
Years since graduation				
<15	15.30±26.53	4.34	0.16	87.54
≥15	6.46±8.15	2.77	0.01	21.99
Post-degree qualifications				
No	-			
Yes	12.70±24.70	2.36	0.01	87.54

Table S3 21 abdominal lymph nodes samples from cats with suspected lymphoma, processed or not for flow cytometry, according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	4	1
>5 cm	3	2
Sample catching		
Blind aspiration	0	0
Ultrasound-guided	9	5
Pharmacological restraint		
None	2	2
Mild sedation	5	1
General anaesthesia	1	0
Needle size		
18 G	1	0
22 G	6	1
23 G	2	1
Sampling technique		
Fine needle aspiration	8	3
Fine needle capillary biopsy	4	0
Transport tube		
Saline solution	0	0
Culture medium	10	2
Years since graduation		
<15	3	2
≥15	6	1
Post-degree qualifications		
No	1	0
Yes	5	3

Table S4 cellular concentration of 21 abdominal lymph nodes samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Lesion size				
<2 cm	1.58			
2-5 cm	1.90±1.61	1.38	0.30	4.40
>5 cm	3.63±4.15	2.37	0.07	10.03
Sample catching				
Blind aspiration	-			
Ultrasound-guided	2.01±2.76	1.16	0.03	10.03
Pharmacological restraint				
None	2.05±2.26	1.26	0.34	5.35
Mild sedation	3.64±3.71	2.48	0.63	10.03
General anaesthesia	0.3			
Needle size				
18 G	3.39			
22 G	7.69±3.31	7.69	5.35	10.03
23 G	1.56±1.31	1.56	0.63	2.48
Sampling technique				
Fine needle aspiration	2.67±3.41	1.34	0.30	11.58
Fine needle capillary biopsy	4.61±4.04	3.51	1.38	10.03
Transport tube				
Saline solution	-			
Culture medium	2.75±2.97	1.63	0.30	10.03
Years since graduation				
<15	4.50±4.17	3.51	0.93	10.03
≥15	1.44±1.16	1.38	0.3	3.39
Post-degree qualifications				
No	5.35			
Yes	2.32±3.45	1.38	0.3	10.03

Table S5 21 thoracic lymph node/masses samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	1	0
2-5 cm	6	0
>5 cm	4	3
Sample catching		
ultrasound-guided	11	3
computed tomography-guided	2	2
Pharmacological restraint		
None	4	1
Mild sedation	6	0
General anaesthesia	2	3
Needle size		
18 G	1	0
22 G	6	2
23 G	3	0
25 G	1	0
Sampling technique		
Fine needle aspiration	10	2
Fine needle capillary biopsy	4	2
Transport tube		
Saline solution	1	0
Culture medium	11	3
Years since graduation		
<15	5	1
≥15	4	1
Post-degree qualifications		
No	2	1
Yes	5	1

Table S6 cellular concentration of 21 thoracic lymph node/masses samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	1.99			
2-5 cm	18.99±23.50	7.88	3.16	59.26
>5 cm	18.46±24.58	5.86	0.01	58.02
Sample catching				
ultrasound-guided	12.64±21.12	1.99	0.61	59.26
computed tomography-guided	15.94±21.14	7.88	0.01	39.93
Pharmacological restraint				
None	13.86±24.71	3.16	1.71	58.02
Mild sedation	18.51±24.04	10.00	1.51	59.26
General anaesthesia	10.41±19.68	0.85	0.01	39.93
Needle size				
18 G	59.26			
22 G	10.00±19.65	2.44	0.01	58.02
23 G	1.75±0.34	1.75	1.51	1.99
25 G	20.2			
Sampling technique				
Fine needle aspiration	10.61±19.80	1.59	0.01	59.26
Fine needle capillary biopsy	16.70±24.44	1.99	1.58	58.02
Transport tube				
Saline solution	1.99			
Culture medium	10.37±18.96	1.65	0.01	59.26
Years since graduation				
<15	12.41±22.62	2.44	1.58	58.02
≥15	13.56±25.59	1.99	0.61	59.26
Post-degree qualifications				
No	42.43±28.09	58.02	10.00	59.26
Yes	2.30±1.50	1.71	0.61	4.44

Table S7 17 effusion samples from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Sample catching		
Blind aspiration	1	0
ultrasound-guided	3	3
computed tomography-guided	3	0
Pharmacological restraint		
None	1	1
Mild sedation	1	1
General anaesthesia	4	0
Needle size		
20 G	1	0
21 G	3	1
22 G	2	1
Sampling technique		
Fine needle aspiration	5	0
Fine needle capillary biopsy	1	1
Years since graduation		
<15	2	0
≥15	3	2
Post-degree qualifications		
No	0	0
Yes	4	0

Table S8 cellular concentration of 17 effusion samples from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	Median	Minimum	Maximum
Sample catching				
Blind aspiration	89.88			
ultrasound-guided computed tomography-guided	31.95±28.56	37.75	0.47	66.04
	12.50±14.86	4.75	3.11	29.63
Pharmacological restraint				
None	70.22±27.81	70.22	50.55	89.88
Mild sedation	21.35±23.20	21.35	4.94	37.75
General anaesthesia	12.50±14.86	4.75	3.11	29.63
Needle size				
20 G	21.03			
21 G	49.61±36.72	51.90	4.75	89.88
22 G	27.75±32.25	27.75	4.94	50.55
Sampling technique				
Fine needle aspiration	19.97±26.76	4.94	3.11	66.04
Fine needle capillary biopsy	50.55			
Years since graduation				
<15	4.94			
≥15	53.05±26.41	50.55	21.03	89.88
Post-degree qualifications				
No	-			
Yes	30.67±31.67	21.03	4.94	66.04

Table S9 14 different samples (skin, kidney, spleen, liver, urine) from cats with suspected lymphoma, processed or not for flow cytometry according to different pre-analytical factors

	Number of samples	
	Processed	**Not processed**
Lesion size		
<2 cm	3	0
2-5 cm	1	3
Sample catching		
ultrasound-guided	3	3
Surgical access	0	1
Catheterisation	1	0
Pharmacological restraint		
None	2	1
Mild sedation	1	0
General anaesthesia	2	0
Needle size		
18 G	0	2
22 G	2	1
23 G	1	0
Sampling technique		
Fine needle aspiration	5	4
Fine needle capillary biopsy	2	0
Transport tube		
Saline solution	1	0
Culture medium	3	3
Years since graduation		
<15	1	1
≥15	4	2
Post-degree qualifications		
No	0	0
Yes	2	1

Table S10 cellular concentration of 14 different samples (skin, kidney, spleen, liver, urine) from cats with suspected lymphoma, sent to the laboratory for flow cytometric immunophenotyping, according to different pre-analytical factors

	Cellularity (x 10^3 cells / µl)			
	Mean ± SD	**Median**	**Minimum**	**Maximum**
Lesion size				
<2 cm	2.49±1.15	2.49	1.67	3.30
2-5 cm	4.78±6.65	2.34	0.03	14.40
Sample catching				
ultrasound-guided	0.74±0.64	0.66	0.02	1.67
Surgical access	4.01			
Catheterisation	14.40			
Pharmacological restraint				
None	1.45±1.68	1.02	0.02	3.30
Mild sedation	1.67			
General anaesthesia	14.40			
Needle size				
18 G	2.02±2.81	2.02	0.03	4.01
22 G	1.66±2.32	1.66	0.02	3.30
23 G	1.67			
Sampling technique				
Fine needle aspiration	4.80±8.45	1.67	0.02	26.89
Fine needle capillary biopsy	3.30			
Transport tube				
Saline solution	3.30			
Culture medium	3.51±6.13	1.45	0.02	14.40
Years since graduation				
<15	0.02			
≥15	4.07±5.27	2.49	0.03	14.40
Post-degree qualifications				
No	-			
Yes	0.52±0.71	0.52	0.02	1.02