1 **Original article**
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3 **Effects of pre-analytical variables on flow cytometric diagnosis of canine lymphoma: A**
4 **retrospective study (2009-2015)**
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19 **Abstract**

20 An increasing number of veterinarians use flow cytometry (FC) for immunophenotyping and
21 staging of canine lymphoma, since it is minimally invasive and cost effective. The aim of this
22 retrospective study was to assess pre-analytical variables that might influence the diagnostic utility
23 of FC of lymph node (LN) fine needle aspirate (FNA) specimens from dogs with
24 lymphoproliferative diseases. The database of the Department of Veterinary Medicine, University
25 of Milan, Italy, from 2009 to 2015 was interrogated. Cases were selected if a LN FNA sample was
26 sent for immunophenotyping and if the final FC report was available. Cases were grouped into
27 'diagnostic' and 'non-diagnostic'. The following pre-analytical factors were analysed by univariate
28 and multivariate analysis: patient-related factors (breed, age, sex, size), operator-related factors
29 including sampling and shipping (year, season, shipping method, submitting veterinarian), and
30 sample-related factors (type of sample material, cellular concentration, cytological smears,
31 artefacts).

32

33 A total of 987 cases fulfilled the inclusion criteria. Among all evaluated variables, the
34 submitting veterinarian, sample material, sample cellularity and artefacts affected the likelihood of
35 having a diagnostic sample. The availability of specimens from different sites and of cytological
36 smears increased the odds of obtaining a diagnostic result. Major artefacts affecting diagnostic
37 utility included poor cellularity and the presence of dead cells. Flow cytometry on LN FNA samples
38 yielded conclusive results in more than 90% of cases with adequate sample quality and sampling
39 conditions.

40

41 *Keywords:* Canine lymphoma; Diagnosis; Fine needle aspirate; Flow cytometry; Pre-analytical

42 **Introduction**

43 Lymphoma is the most common haematopoietic malignancy in dogs, with an incidence of
44 about 84 cases/100,000 dogs per year (Dorn et al., 1970). Classification schemes, and diagnostic
45 and therapeutic approaches tend to reproduce those applied in human medicine, with only a few
46 exceptions, including a higher prevalence of multicentric lymphomas and diffuse versus nodular
47 lymphoma (Marconato et al., 2013a). These features favour the use of minimally invasive
48 diagnostic techniques such as fine needle aspirate (FNA) cytology and subsequent flow cytometry
49 (FC) (Comazzi and Gelain, 2011).

50

51 Fine-needle aspiration and cytology is now considered to be the first diagnostic approach for
52 canine lymphoma (Marconato et al., 2013a), since it is cost-effective, minimally invasive and well
53 accepted by the owners. The information provided by cytology in association with other ancillary
54 techniques sets the basis for therapeutic decisions in the majority of cases, as reported by a recent
55 survey (Regan et al., 2013). Recently, the use of FC has significantly increased in veterinary
56 medicine due to the increased number of canine-specific monoclonal antibodies available. Flow
57 cytometry is routinely used for immunophenotyping of lymphoma and to refine the diagnosis of
58 specific lymphoma subtypes (Seelig et al., 2014; Martini et al., 2015). It can also be used for
59 lymphoma staging (Marconato et al., 2013b) and to evaluate minimal residual disease after
60 treatment (Aresu et al., 2014). However, veterinary FC facilities are currently limited to a few
61 reference laboratories, mainly due to economic reasons. Most of them are part of academic
62 institutions and provide services for veterinary hospitals, veterinary laboratories and private
63 practices. The specific requirements for sample preparation and shipping may partially limit the
64 utility when FC is not readily available and could also bias results, even though specific studies are
65 still lacking.

66

67 Since 2008, the FC facility of the authors' institutions offer a reference service for canine
68 lymphoma and leukaemia flow cytometry, receiving samples from veterinary oncologists working
69 in the same institution and external veterinarians from Italy, Switzerland, Portugal, Spain, Slovenia
70 and the UK. Given our experience, we hypothesised that different sampling techniques, shipping
71 and storage conditions might bias the results and influence the diagnostic performance of FC. To
72 the authors' knowledge, only one study on the influence of pre-analytical variables on diagnostic
73 performance of FC is available in cats (Martini et al., 2017), but a study evaluating the effects of
74 such variables in a high number of dogs is still lacking.
75
76 The aim of this retrospective study was to assess whether and how pre-analytical variables
77 might influence the diagnostic utility of lymph node (LN) FNA samples obtained from dogs with
78 clinically suspected lymphoproliferative disease analysed by FC. The goal was to create
79 recommendations for sampling techniques, sample storage and shipping in order to decrease pre-
80 analytical errors and to increase the diagnostic utility of FC for the diagnosis of lymphoma and
81 leukaemia in dogs.
82

83 **Material and methods**

84 *Inclusion criteria*

85 The FC database of the Department of Veterinary Medicine, University of Milan, Italy, was
86 searched retrospectively and canine cases were identified over a period of six years (2009-2015).
87 Inclusion criteria were: (1) LN FNA sent for flow cytometric immunophenotyping; and (2) presence
88 of a final FC report in the database. If other sample types, such as peripheral blood (PB), bone
89 marrow (BM) aspirates, body cavity effusions, FNA from mass lesions, spleen, liver or other
90 tissues, were submitted, these cases were included in the present caseload only if a LN FNA from
91 the same patient was analysed, regardless of the diagnostic pathway used and what tissue (e.g. PB
92 or BM for leukaemias) was considered first in the diagnostic pathway. Exclusion criteria included:

93 (1) cases composed of tissues other than LN aspirates; and (2) cases sent for minimal residual
94 disease analysis.
95
96 *Sample collection*
97 Samples were collected from one or more enlarged LNs by multiple aspirations, with or
98 without suction, using a fine needle (21-22 ga). The collected material was suspended in 1 mL of a
99 transport medium (Roswell Park Memorial Institute, RPMI 1640, recommended; phosphate
100 buffered saline; saline solution, not recommended), refrigerated at 4-6 °C and shipped to the
101 laboratory within 24 h from sampling with a cold pack in the envelope to maintain sample cooling.
102
103 At admission, samples were analysed visually and cellularity was evaluated using an
104 automated analyser (Sysmex XT-2000iV). Cellularity was generally considered to be suitable if the
105 cell concentration in 1 mL was > 5 x 10^9 cells/L (corresponding to > 5 x 10^6 cells in total), whereas
106 samples with < 1 x 10^9 cells/L (corresponding to < 1 x 10^6 cells in total) were generally excluded
107 from processing, although slight variability may have occurred due to the preferences of the
108 operator dealing with the sample.
109
110 *Flow cytometry*
111 Processing for FC was performed as described previously (Gelain et al., 2008). The
112 erythrocyte lysis step was generally not considered necessary for LN FNA samples unless gross
113 haemodilution was detected at visual inspection. For PB and BM samples, red blood cells were
114 lysed by adding a lysis solution containing 8% ammonium chloride.
115
116 The panel of antibodies applied to LN samples was adapted over the years due to the
117 increased availability of conjugated antibodies against canine leukocytes. Samples processed before
118 2011 were analysed using mainly a two-colour approach. Starting from 2011, a multicolour

119 approach was applied with the addition of CD45 as tracking label in all tubes. The diagnostic
120 algorithm varied throughout the years, but a basic panel included antibodies against CD5, CD21,
121 CD34, and CD45. On the basis of the staining results obtained with this panel, expression of other
122 antigens was evaluated, including CD3, CD4, CD8, CD11b, CD14, CD18, CD20, CD25, CD44,
123 CD79a, CD117 and MHCII. The antibody panel used to label PB and BM samples varied based on
124 the phenotype of neoplastic cells identified in the LN sample. If the LN sample was not adequate
125 for FC, the basic antibody panel was applied to PB and BM, with the addition of CD4 and CD8.
126 Specificity, sources and clones of antibodies are listed in Novacco et al. (2015). Samples were
127 acquired using a flow cytometer (FACScalibur, Becton Dickinson) and analysed using Cell Quest
128 software (Becton Dickinson). All FC interpretations and reports were made by one board-certified
129 clinical pathologist (SC).
130
131 The final diagnosis was based on a comprehensive evaluation of all the data provided by the
132 referring veterinarian, including different combinations of history, presenting complaint, clinical
133 signs, LN cytological smear, haematology data and LN/PB/BM FC analysis. Cases included in the
134 present study were classified based only on the FC report, without any re-evaluation of the raw FC
135 data.
136
137 *Criteria for diagnosis*
138 The final diagnosis was derived from the flow cytometry report and was based on cytology
139 findings, if available, combined with a comprehensive interpretation of flow cytometric results
140 obtained from LN aspirates and other sample material. Samples were considered to be 'non-
141 diagnostic' if one or more of the following criteria were present: (1) acellularity ($<1 \times 10^6$ cells in
142 total); (2) sample composed of primarily dead cells; and (3) if flow cytometric results strongly
143 differed from cytological evaluation, for example, when neoplastic cells disrupted during
144 processing and only a small residual non-neoplastic population was labelled). Dead cells were

145 identified by gross evaluation of the sample (based on colour, odour or the presence of tissue
146 debris) and/or with a viability stain (propidium iodide), which was included in the last 2 years of the
147 study period. Samples were considered 'negative for lymphoid tumour' if: (1) a mixed population
148 of predominantly small cells was present with a cytology supporting a reactive/hyperplastic lymph
149 node; or (2) other causes of lymphadenomegaly were identified by immunophenotyping and
150 cytology, for example, LN metastasis from solid tumours, histiocytic tumours and plasma cell
151 tumours. Samples were considered as 'likely lymphoid tumour' if: (1) flow cytometry from a lymph
152 node showed a highly prevalent population (> 65%) of lymphoid cells with a single phenotype, but
153 only a reduced panel of antibodies was allowed and/or the lack of a good quality cytological smear
154 precluded a definitive diagnosis; (2) flow cytometry from a lymph node was poorly cellular ($< 1 \times$
155 10^6 cells in total) or provided equivocal results, but LN cytology was highly suggestive of
156 lymphoma and immunophenotyping of PB and/or BM was suggestive of a lymphoid tumour. In
157 these cases, a diagnosis of 'likely lymphoid tumour' was made. Cases were classified as 'lymphoid
158 tumour' if a definitive diagnosis of lymphoma or leukaemia was made based on the results of
159 immunophenotyping of LN, PB, or BM. In many cases, extended subtyping of lymphoid neoplasia
160 was possible based on immunophenotyping of different tissues and cytological evaluation: (1) B
161 cell lymphoma (irrespectively of the grade); (2) high grade T cell lymphoma (based on
162 immunoreactivity to T cell markers and cytological aspects including high numbers of mitotic
163 figures); (3) low grade T cell lymphoma (based on typical T zone pattern staining on FC and/or
164 distinctive cytological features); (4) acute leukaemia (starting with PB or BM immunophenotyping
165 of precursor cells confirmed by LN infiltration); and (5) chronic lymphocytic leukaemia (starting
166 with PB or BM immunophenotyping confirmed by LN infiltration).
167
168 *Statistical analysis*
169 Statistical analysis was applied to identify pre-analytical variables possibly affecting the
170 likelihood to reach a diagnosis; for this aim, samples were grouped into 'diagnostic' (if any

diagnosis had been made, regardless of the level of confidence) and 'non-diagnostic' (including samples not adequate for processing and samples processed but not diagnostic). Pre-analytical variables investigated were related to: (1) the patient: breed (pure or mixed), age (years), sex (female, spayed female, male, neutered male), size (toy/small, medium, large/giant); (2) sampling and shipping: year (from 2009 to 2015), season (cool to cold from October to March, warm to hot from April to September), referring veterinarian (veterinarians who sent > 10 cases over the study period were considered individually, while veterinarians sending less than 10 cases in total were grouped in a single category), origin and shipping ('within institution', 'out of institution hand-delivered', 'out of institution delivered by express courier'); (3) sample: type of sample (LN alone, LN plus PB, LN plus PB and BM, LN plus BM), LN cytological smear (present or not), cellular concentration of the LN sample (x 10^9 cells/L), presence of gross artefacts (haemodilution, dead cells, none). Univariate and multivariate binomial logistic regressions were performed using SPSS v20.0 for Windows (IBM). Multivariate analysis was performed with a backward step selection, including only variables with $P \leq 0.3$ at univariate analysis. Significance was set at $P \leq 0.05$.

Results

Out of 1273 samples, 264 cases were excluded due to lack of information regarding the sample (n = 88; 6.9%) or due to sampling of tissues other than LN (n = 176; 13.8%). Among the latter, 59 (33.5%) cases were represented by PB and BM, 49 (27.8%) cases by PB alone, 28 (15.9%) cases by extranodal masses (other than cutaneous), 18 (10.2%) cases by effusions, nine (5.1%) cases by spleen, seven (4.0%) cases by cutaneous masses, two (1.1%) cases by liver, and four (2.3%) cases by other tissues. Twenty-two cases (1.73%) were excluded because the final report was not available. Finally, 987 cases fulfilled the inclusion criteria and were included in the statistical analysis.

196 Seventy-two (7.3%) samples were non-diagnostic. Among the 915 diagnostic FNAs, 839
197 (91.7%) were ‘conclusive for lymphoid tumour’, 61 (6.7%) were ‘negative for lymphoid tumour’,
198 and 15 (1.6%) were ‘likely lymphoid tumour’. A specific diagnosis was available for 812 cases; 596
199 (73.4%) cases were B cell lymphomas, 143 (17.6%) cases were high grade T cell lymphomas, 60
200 (7.4%) cases were low grade T cell lymphomas, 8 (1.0%) cases were acute leukaemias, and five
201 (0.6%) cases were chronic lymphocytic leukaemias. The results of univariate and multivariate
202 analyses and the odd ratios for diagnostic samples are summarised in Table 1.
203
204 *Patient-related variables*
205 Breed was reported in 911 cases; there were 239 (26.2%) mixed breed dogs. Among the
206 remaining 672 dogs, the most prevalent breeds included German shepherd (*n* = 59; 8.8%), Boxer (*n*
207 = 56; 8.3%), Golden retriever (*n* = 46; 6.8%), Labrador retriever (*n* = 44; 6.5%), Rottweiler (*n* = 38;
208 5.7%) Doberman pinscher (*n* = 37; 5.5%), Beagle (*n* = 29; 4.3%), Bernese mountain dog (*n* = 23;
209 3.4%), English bulldog (*n* = 21; 3.1%) and Yorkshire terrier (*n* = 21; 3.1%). Many other breeds
210 were represented, with less than 20 cases each. In 76 cases, the breed was not recorded. Since the
211 size of mixed breed dogs was generally not available, this variable was analysed only within the
212 pure-breed dog group. Among the latter, the majority of cases were of large/giant size (*n* = 415;
213 61.8%); 142 (21.1%) dogs were of toy/small size, and 115 (17.1%) were medium-sized. Sex was
214 reported in 901 cases: there were 213 (23.6%) females, 210 (23.3%) spayed females, 397 (44.1%)
215 males and 81 (9.0%) neutered males. The male/female ratio was 1.15:1. Age was known for 899
216 dogs. Median age was 8 years (mean 8.4 ± 3.2 years, range 1-17 years). None of the patient-related
217 variables significantly influenced the likelihood of having a diagnostic sample.
218
219 *Variables related to sampling and shipping*
220 The number of cases per year steadily increased during the study period, ranging from 63
221 cases fulfilling the inclusion criteria in 2009 to 200 cases in 2015. The highest percentage of

222 diagnostic cases was reached in 2012 (124/128 cases, 96.9%) and the lowest in 2015 (174/200
223 cases, 87.0%). The likelihood of having a diagnostic sample significantly varied by years both with
224 univariate and multivariate analysis (*P* = 0.031 and *P* = 0.016, respectively).
225
226 The percentage of samples collected in the cold and hot seasons was almost similar (51.2%
227 and 48.8%, respectively) without any significant difference (*P*=0,154).
228 Thirteen veterinarians sent >10 samples, ranging from 13 to 207 samples; veterinarians sending <
229 10 samples were grouped all together, to a final count of 363 (36.8%) cases. Most of the samples
230 for which this information was available had been sent to the laboratory by express courier
231 (559/816, 68.5%), 159 (19.5%) were sampled outside the institution and hand-delivered, and 98
232 (9.9%) were sampled within the institution. The likelihood of a having a diagnostic sample was not
233 influenced by these variables with univariate analysis. However, the proportion of diagnostic
234 samples significantly varied among veterinarians based on multivariate analysis (*P* = 0.004).
235
236 *Variables related to sample material*
237 In the majority of cases (572; 58.0%), samples from all three sites (LN, PB and BM) of the
238 same dog were available; LN and PB were sent in 225 (22.8%) cases, LN alone was submitted in
239 163 (16.5%) cases, and LN and BM were sent to the laboratory in 27 (2.7%) cases. Sample material
240 significantly influenced the likelihood of obtaining a diagnosis (*P* = 0.025). In particular, cases for
241 which both LN and PB or all three sites (LN, PB and BM) were obtained had a higher likelihood of
242 being diagnostic, compared to samples from LN alone (*P* = 0.011 and *P* = 0.006, respectively). The
243 type of sample material also significantly influenced the likelihood of having diagnostic samples at
244 multivariate analysis (*P* = 0.031).
245
246 A LN cytological smear was provided in 634/987 (64.2%) cases, but the quality of these
247 smears was not assessed in the present study. The presence of a cytological smear significantly

248 improved the likelihood of having a diagnostic sample (*P* = 0.009) only at univariate analysis.
249 Lymph node sample cellularity was highly variable among cases, ranging from 0.02 to 436.19 x 10^9
250 cells/L (median 12.44 x 10^9 cells/L, mean 29.21±48.13 x 10^9 cells/L). Lymph node sample
251 cellularity did not affect the likelihood of having diagnostic samples based on univariate analysis
252 (*P*=0.104) , but was statistically significant at multivariate analysis (*P*=0.013), with cellularity being
253 higher in diagnostic than in non-diagnostic samples (median 13.1 x 10^9 cells/L and 2.09 x 10^9
254 cells/L, respectively). In the group of non-diagnostic cases, the cellularity was lower than the
255 minimum adequate concentration (5.0 x 10^9/L) in 52.8% of cases, and lower than the lower cut-off
256 value generally considered to be suitable for analysis (1.0 x 10^9/L) in 37.5% of cases. These
257 percentages were much higher than those found in diagnostic samples (21.0% and 3.4%,
258 respectively).
259
260 Artefacts were reported in nine non-diagnostic samples (12.5%), including one
261 haemodiluted sample and eight necrotic samples, and in 18 diagnostic samples (2.0%), including
262 three haemodiluted and 15 necrotic samples. Haemodilution did not affect the likelihood of having
263 a diagnostic sample. On the contrary, necrotic samples were significantly less likely to be diagnostic
264 in both univariate and multivariate analysis ($P < 0.001$ for both analyses).
265

266 Discussion

267 The results of the present study indicate that FC is suitable to refine a tentative diagnosis of
268 canine lymphoma because it provided diagnostic results in the majority of cases (about 90%).
269 Among the pre-analytical variables investigated, no association was identified between signalment
270 and the likelihood to obtain a diagnostic result. In particular, neither dog size nor breed (often
271 related to breed specific temperament) influenced the odds of obtaining a diagnosis. Fine needle
272 aspiration of a lymph node is usually carried out without sedation, and, in theory, obtaining a good
273 quality fine needle aspirate is more difficult in small-sized or aggressive dogs. On the other hand,

274 the sampling of superficial lymph nodes may be more difficult in breeds in which the subcutaneous
275 tissue is abundant. However, the results of the present study indicate that FNA provides good
276 quality samples in the great majority of cases regardless of the animal's signalment.
277
278 In contrast, the likelihood of obtaining a diagnosis varied among submitting
279 veterinarians/institutions, possibly reflecting different technical skills of the operator (veterinarian)
280 who performs the sampling. These data support the necessity of an adequate technical training in
281 order to increase the likelihood of having a diagnostic result.
282
283 Delivery method did not influence the diagnostic utility. No differences were found among
284 samples submitted from the internal oncology service of the University of Milan, which were
285 delivered within few minutes following sampling, those delivered in person within a few hours
286 following sample collection, and those shipped using an express courier.
287
288 Likewise, no differences were found between samples shipped during the cold and hot
289 seasons. Of notice, the standard requirements of our laboratory include a maximum shipping time of
290 24 h and shipment on ice packs for FC samples. Therefore, our results are only valid under these
291 conditions. In human medicine, refrigeration of FC samples generally is not recommended, since
292 some antigens may be internalised and bias the final results (Ekong et al., 1993). Studies on this
293 aspect in veterinary medicine are currently lacking, but it seems reasonable to refrigerate the
294 samples during shipment to prevent cell disruption due to high temperature, mainly in hot seasons
295 and in hot countries. It is currently unknown whether canine antigens vary their expression
296 following refrigeration.
297
298 Sample type and sample characteristics had the greatest influence on the diagnostic power of
299 flow cytometry. The major factor leading to a non-diagnostic result was poor cellularity, with more

300 than a half of non-diagnostic samples having a cell concentration lower than recommended.
301 Artefacts such as haemodilution and dead cells showed a minor effect on non-diagnostic samples.
302 Haemodilution was rarely related to a non-diagnostic sample because a rapid lysis step was added
303 to the procedure when gross haemodilution was evident. The presence of many non-viable cells is
304 uncommon in LN FNAs, at least in untreated dogs. When present, the necrotic debris may be easily
305 identified by gross evaluation (based on colour, odour or the presence of tissue debris) or using a
306 viability stain, such as propidium iodide. The latter procedure was included in the last 2 years of the
307 study period but it is not possible to determine if this inclusion could have influenced the percentage
308 of diagnostic results.
309
310 Having sample material from different sites (LN, PB, BM) and a concurrent LN cytological
311 smear available improved the likelihood of establishing a diagnosis of lymphoid neoplasia.
312 Therefore, complete cases, including FNA from a LN, PB, BM and cytology smears, are desirable
313 for a more comprehensive evaluation by a clinical pathologist. Specifically, cytological evaluation
314 of a smear may help to differentiate reactive/hyperplastic lymph nodes from lymphoid tumours in
315 which the neoplastic population, which tends to be more fragile, has been disrupted. In the first
316 case, samples would be classified as 'diagnostic, non-neoplastic', while in the second case they
317 would be classified as 'non-diagnostic'. When a fresh cytological smear was not included, a
318 cytospin or a cytology of the sediment from the cell suspension submitted for FC was generally
319 prepared to better interpret FC results. However, these smears were often of poor quality for an
320 adequate cytological evaluation and they were only used to confirm the FC results.
321
322 The presence of neoplastic cells detected via flow cytometry in PB and/or BM, together with
323 a LN cytology compatible with lymphoma, may also permit a putative diagnosis of 'likely
324 lymphoma', even if LN sample cellularity is too low for FC. This could be achieved when
325 neoplastic cells can be differentiated from non-neoplastic cells, for example when the cells showed

326 an aberrant phenotype (such as a T zone pattern) or when a high percentage of large CD21 positive
327 cells were detected. Although it cannot be presumed that the cells in peripheral blood represented
328 the same neoplastic population than the cells in lymph node, we considered it very likely that they
329 were of similar origin when a cytological smear of lymph node was suggestive of lymphoma. In
330 such cases , we used a comprehensive approach and considered all available clinical and laboratory
331 information to make a diagnosis of 'likely lymphoma'. Although this assumption should be taken
332 with care, we think that the stepwise approach of including FC of PB and/or BM when the LN
333 sample is not suitable could be of some clinical benefit in managing dogs with lymphoma. The
334 evaluation of PB and/or BM would also be crucial to differentiate acute and chronic lymphoid
335 leukaemias with secondary lymph node infiltration from nodal lymphoma with a haematogenous
336 phase. The results of our study suggest that, when LN cellularity was too low to perform FC, flow
337 cytometry of PB was most likely to be diagnostic in case it was infiltrated. This suggests that
338 submitting peripheral blood together with a LN aspirate will facilitate interpretation of FC results
339 and it will also assist with staging of lymphoma. In the present study, PB and BM samples were
340 frequently submitted to our laboratory in addition to a LN sample, mainly for staging purposes. Our
341 results indicate that having a BM aspirate available for FC did not increase the likelihood of having
342 a diagnostic sample if LN and PB were also submitted.

343

344 The purpose of this study was not to assess the diagnostic accuracy of FC in identifying
345 different lymphoma subtypes. However, the frequency of lymphoma subtypes reported here is
346 similar to frequencies reported in the literature (Ponce et al., 2010; Valli et al., 2011). This finding
347 suggests that FC may be useful to identify different subtypes in dogs with lymphoma. This may be
348 attributable to the high prevalence of diffuse lymphomas in dogs (Fournel-Fleury et al., 1994,
349 Comazzi and Gelain, 2011).

350

351 The limits of this study are inherent to its retrospective nature. Some possible sources of
352 biases were not analysed, including the localisation and size of the LN sampled, the technique used
353 for sampling (free hand vs. ultra-sound guided, suction vs. capillary technique), the use of different
354 transport media (RPMI vs. phosphate buffer saline vs. saline solution), and the quality of the
355 cytological smears. Another limitation is the lack of a gold standard for the final diagnosis, which
356 precluded us from defining the diagnostic performances of both cytology and FC. This should be
357 addressed in a future, prospective study.

358

359 **Conclusions**

360 Flow cytometry performed from canine LN FNA samples is a suitable diagnostic tool to
361 confirm lymphoma in the majority of cases, regardless of patient characteristics, provided that the
362 sample has sufficiently high cellularity, is submitted within 24 h following sampling and is shipped
363 on ice packs. Packaging and shipping should be standardised to assure fast delivery within 24 h of
364 sampling. Haemodilution has minimal effect on FC analysis, but veterinarians should make any
365 possible effort to obtain a highly cellular sample. The centres of large, neoplastic nodes should be
366 avoided if possible, they may provide more necrotic/dead cells. The addition of a PB sample and a
367 LN cytological smear can be recommended to improve the likelihood of receiving a conclusive
368 result.

369

370 **Conflict of interest statement**

371 None of the authors has any other financial or personal relationships that could
372 inappropriately influence or bias the content of the paper.

373

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431 **Table 1**
432 Results of univariate and multivariate analysis for different pre-analytical variables examined for their effect on flow
433 cytometric analysis of samples from dogs with clinically suspected lymphoma.
434

	Pre-analytical variable		Univariate analysis			Multivariate analysis
			Odds ratio	95% confidence interval	*P* value	*P* value
Related to animal	Breed		0.999	0.597-1.670	0.996	ND
	Age		1.035	0.959-1.117	0.378	ND
	Sex	Neutered male	Ref	-	0.330	ND
		Male	2.672	0.803-8.888		
		Female	2.112	0.599-7.450		
		Spayed female	1.857	0.519-6.641		
	Size	Toy/Small	Ref	-	0.609	ND
		Medium	1.032	0.429-2.482		
		Large/giant	0.754	0.371-1.531		
Related to operator	Year	2009	Ref	-	0.031 *	0.026 *
		2010	0.681	0.174-2.664		
		2011	0.907	0.227-3.631		
		2012	1.550	0.336-7.146		
		2013	0.562	0.153-2.066		
		2014	0.775	0.212-2.839		
		2015	0.335	0.098-1.145		
	Season	Cold	Ref	-	0.154	0.130
		Hot	0.703	0.433-1.142		
	Veterinarian				0.067	0.020 *
	Origin/shipping method	Express courier	Ref	-	0.247	0.590
		Hand-delivered	0.714	0.353-1.446		
		Within institution	0.453	0.160-1.286		
Related to sample	Sample material	LN alone	Ref	-	0.025 *	0.049 *
		LN and PB	2.877	1.346-6.150		
		LN, PB and BM	2.078	1.182-3.653		
		LN and BM	1.849	0.408-8.380		
	LN cytological smear	No	Ref	-	0.009 *	0.313
		Yes	1.904	1.176-3.083		
	Dead cells	No	Ref	-	< 0.001 *	< 0.001 *
		Yes	0.133	0.054-0.326		
	Haemodilution	No	Ref	-	0.210	ND
		Yes	0.234	0.024-2.274		
	LN sample cellularity		1.008	0.998-1.017	0.104	0.013 *

435
436 LN, lymph node; PB, peripheral blood; BM, bone marrow; Ref, reference; ND, not detectable.

437 Odds ratio for the probability to provide a diagnostic result are also given.

438 * $P \leq 0.05$

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