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Abstract

The most promising techniques for detecting minimal residual disease (MRD) in canine lymphoma are flow cytometry (FC) and polymerase chain reaction amplification of antigen receptor genes (PARR). However, the agreement between these methods has not been established. MRD was monitored by FC and PARR following treatment of dogs affected with diffuse large B-cell lymphoma (DLBCL), comparing results in lymph node (LN), peripheral blood (PB) and bone marrow (BM) samples. The prognostic impact of MRD on time to relapse (TTR) and lymphoma-specific survival (LSS) was also assessed.

Fourteen dogs with previously untreated DLBCL were enrolled into the study; 10 dogs eventually relapsed, while four dogs with undetectable MRD were still in remission at the end of the study. At diagnosis, the concordance rate between FC and PARR was 100%, 78.6%, and 64.3% for LN, PB and BM, respectively. At the end of treatment, the agreement rates were 35.7%, 50%, and 57.1% for LN, PB and BM, respectively. At least one of the follow-up samples from dogs experiencing relapse was $PARR^{+}$; conversely, FC was not able to detect MRD in seven of the dogs that relapsed. PARR was more sensitive than FC in predicting TTR, whereas the combination of PARR and FC was more sensitive than either technique alone in predicting LSS using PB samples. The results suggest that immunological and molecular techniques should be used in combination when monitoring for MRD in canine DLBCL.

Keywords

- Dog; Lymphoma; Minimal residual disease; Flow cytometry; PARR

Introduction

Development of better treatments for diffuse large B-cell lymphoma (DLBCL) in dogs has resulted in improvements in terms of prognosis (Garrett et al, 2002 and Simon et al, 2006). However, following chemotherapy, the continued presence of malignant cells, representing minimal residual disease (MRD), places dogs affected with DLBCL at risk of relapse and consequently treatment is rarely curative (Marconato et al., 2011). Therefore, challenges remain in terms of detecting and eradicating residual tumour cells completely, thereby improving outcome. Lymph node (LN), bone marrow (BM) and peripheral blood (PB) involvement in lymphoma has traditionally been detected by cytology and flow cytometry (FC) (Marconato et al, 2013 and Martini et al, 2013); however, if the burden of malignant cells is low, FC might not be sufficiently sensitive to detect some cases with MRD (Merli et al., 2010).

In recent years, molecular techniques have been introduced for diagnostic purposes (Burnett et al., 2003), for molecular staging at diagnosis (Lana et al, 2006 and Sato et al, 2013) or for monitoring during chemotherapy (Yamazaki et al, 2008 and Yamazaki et al, 2010; Sato et al, 2011 and Sato et al, 2013; Gentilini et al., 2013). Detection of MRD using PB samples, taken following treatment, can be used to judge treatment efficacy and to select dogs that might benefit from consolidation chemotherapy, despite complete remission (CR) as determined clinically (Yamazaki et al, 2010 and Sato et al, 2011). While all these studies have analysed circulating neoplastic cells, only one study focused on residual disease in LN aspirates (Thilakaratne et al., 2010), finding no significant correlation between MRD detection in PB and LN.

MRD can be determined in canine B-cell lymphoma by FC and polymerase chain reaction amplification of antigen receptor genes (PARR). The immunoglobulin heavy chain (IgH) genes represent the most useful molecular target for detecting B-cell clonality, since they rearrange during early lymphoid development and demonstrate extensive junctional diversity (Mitterbauer-Hohendanner et al., 2004). The aims of the present study were to determine the presence of rearranged IgH genes in LN, BM and PB samples from dogs affected with DLBCL, following treatment, to compare this technique to FC. Moreover, the study was designed to assess whether LN, PB or BM were equivalent, as tissue sources to detect MRD and also to determine the prognostic impact of MRD, determined by FC and PARR, on time to relapse (TTR) and lymphoma-specific survival (LSS).

Material and methods

Animals and inclusion criteria

Client-owned dogs, with recently diagnosed, histologically confirmed DLBCL, were prospectively enrolled. To be eligible for recruitment, dogs were required to undergo complete staging (Marconato et al., 2013), including FC on LN, PB and BM aspirates and histopathological evaluation of the previously aspirated LN, which was surgically removed (further information in Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data). Written, informed consent was obtained from all owners and the study conformed to Italian law on use of animals in research (D. Lgs. n. 116/92).

Treatment and evaluation of treatment response

All dogs were enrolled in a therapeutic trial and received dose-intense chemotherapy, consisting of L-asparaginase, vincristine, cyclophosphamide, doxorubicin, lomustine, and prednisone (details of the protocol in Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data), with some of the dogs also receiving an autologous vaccine (Marconato et al., 2014). The

response to treatment was classified as CR, partial remission (PR), stable disease, or progressive disease (PD), based on previously published criteria (Vail et al., 2010). Responses were required to last for at least 28 days. Relapse was defined as clinical reappearance and cytological evidence of lymphoma at any anatomical site, in dogs having experienced CR, whereas relapse for animals with PR was defined as progression of disease. Response was evaluated at each chemotherapy session by measurement of peripheral LN.

End-staging was carried out at the end of chemotherapy (T1), and all clinical, radiological, ultrasonographic, or laboratory investigations that indicated abnormalities at pretreatment staging (T0) were repeated. BM and PB were sampled again in all cases. Fine-needle aspiration of any peripheral LN that had been enlarged at diagnosis was carried out; the same LN was then surgically removed (end-staging lymphadenectomy).

Tissue samples

At T0 and T1, samples were collected for analysis. Samples included LN tissue in formalin and RNAlater solution (Life Technologies), fine-needle aspirates of peripheral enlarged LN in RPMI 1640 (Sigma–Aldrich), PB and BM samples in EDTA sample tubes and RNAlater solution. For histopathological examination, 3 μm sections of formalin-fixed and paraffin-embedded LN were stained with haematoxylin and eosin. To confirm the B-cell phenotype, a panel of four antibodies was used (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data). Samples were classified, based on the WHO classification (Valli et al., 2011).

Flow cytometry

FC was performed on LN aspirates, BM and PB at T0 and T1 within 24 h of sampling, to confirm B-cell neoplasia and assess the level of infiltration (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data). For LN samples, 5000 events, representing $CD45^+$ cells, were analysed, with 10,000 $CD45^+$ cells analysed for PB and BM. At T0, LN samples were considered diagnostic for DLBCL when at least 80% of the $CD45^+$ cells were large (i.e. FSC > 400) and $CD21^+$. At T1, LN MRD was considered positive, if at least 1% of large $CD21^+$ cells were detected. If large $CD21^+$ cells were not detected by FC, due to scarce cellularity, LN samples were considered negative for MRD. At T0 and T1, the extent of PB and BM infiltration with malignant cells was reported as the percentage of large $CD21^+$ cells in the total population of $CD45^+$ cells, and was considered positive if >1%.

DNA extraction and PARR

Genomic DNA was extracted from LN, PB and BM using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA concentrations were measured by the use of a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and Qubit fluorometer (Life Technologies). Detection of antigen receptor gene rearrangement was assessed by PCR amplification of the complementarity-determining region 3 (CDR3) of the IgH receptor genes, as previously described (Burnett et al., 2003) (further details in Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data). Samples were considered positive, if one dominant and discrete band was present, indicative of the presence of a monoclonal population of B-cells; whereas samples were considered negative, if no bands, a diffuse smear or a laddering were observed.

The sensitivity of the PCR assay to detect a clonal lymphoid population within a polyclonal cell population was evaluated by mixing a starting amount of 30 ng DNA from a monoclonal cell

population, obtained from a dog at T0 (100% clonal cells), with different amounts of a mixture of genomic DNA from mononuclear cells, obtained from 10 healthy individuals. The limit of detection of the assay required at least 300 pg DNA from the monoclonal cell population, diluted in 29.7 ng of polyclonal DNA.

Statistical analysis

TTR was calculated for dogs in CR or PR from T1 to clinical relapse or progression, respectively. Dogs lost to follow-up, deceased for lymphoma-unrelated causes before PD, or those still in CR at the end of the study, were censored for TTR analysis. Time to progression (TTP) was measured as the interval between the start of treatment and PD. LSS was measured as the interval between the start of treatment and death from lymphoma (Vail et al., 2010). Dogs alive at the end of the study, lost to follow-up or deceased due to lymphoma-unrelated causes were censored for survival analysis.

The Kaplan–Meier method was used to assess the influence of LN, PB and BM infiltration with malignant cells, identified by FC and/or PARR, on TTR and LSS and the univariate association between groups was tested using the log-rank test. Analyses were performed using the SPSS statistical package version 12.0.1. *P*-values < 0.05 were considered to be significant.

Results

Clinical characteristics and outcome

Fourteen dogs with DLBCL were enrolled (clinical details in Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.03.006: Supplementary data). Thirteen dogs achieved CR and the other achieved PR, within 1–2 days after having received L-asparaginase. Ten dogs eventually relapsed, while four dogs were still in CR at the end of the study. Overall, median TTP was 242 days (range, 31–635 days). At data analysis closure, eight dogs had died and six were still alive. Of those that had died, six dogs succumbed to lymphoma, whereas two died for unrelated causes. Median LSS was 362 days (range, 199–636 days).

MRD evaluation by histopathology

At T0, centroblastic DLBCL was diagnosed in all 14 dogs (Fig. 1a, b). At T1, all dogs underwent end-staging lymphadenectomy, indicating that two dogs still had DLBCL, with LN of the remaining 12 dogs showing a distorted architecture, thickening of the peripheral capsule, with a diffuse size and reduced number of germinal centres. A mixture of small, medium and large lymphocytes was present in the cortex, with loss of normal anatomical distribution, admixed with frequent apoptotic cells. The medulla was characterised by an increased number of plasma cells and histiocytes (Fig. 1c, d). Eight of these 12 dogs eventually relapsed.

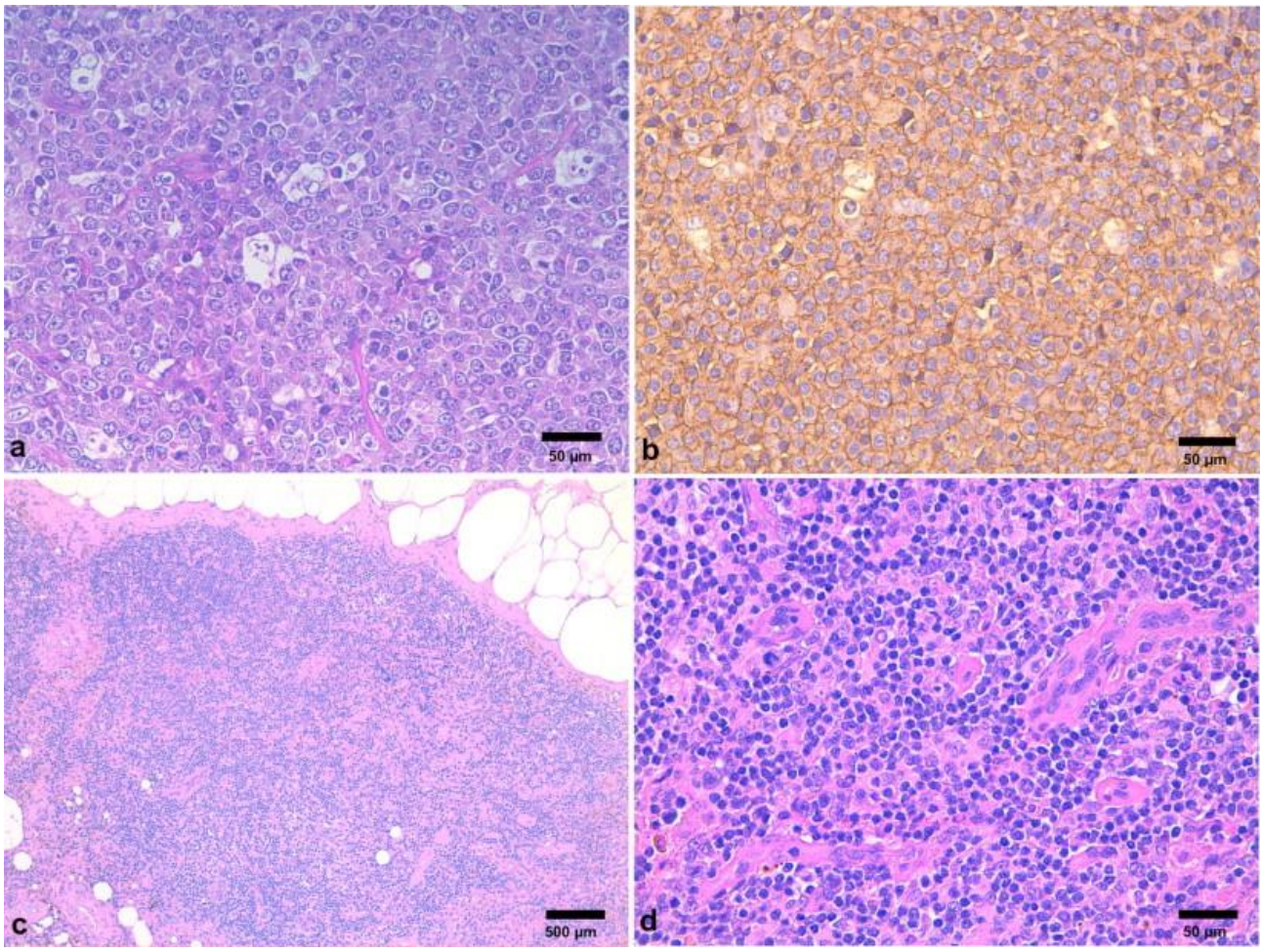


Fig. 1.

(a) Haematoxylin and eosin (HE) staining and (b) immunohistochemistry for CD20 in diffuse large B-cell lymphoma (DLBCL), centroblastic type. (c) HE staining of a surgically removed lymph node after treatment (T1): distorted architecture with thickening of the capsule and reduction of germinal centres. (d) A mixture of small, medium and large lymphocytes is present admixed with frequent apoptotic cells.

MRD evaluation by flow cytometry

At T0, DLBCL was diagnosed in all cases by FC analysis of LN. Malignant cells were identified in PB in seven cases, with a median percentage of 10.7% (range, 2–42%). BM infiltration was identified by FC in nine cases, with a median percentage of 7.2% (range, 1.2–60.7%). Six dogs showed simultaneous infiltration of PB and BM, whereas four did not show evidence of infiltration in either sample. At T1, 10 dogs were classified as being negative for MRD in all samples analysed. At T1, two dogs had detectable MRD in LN, and in the remaining 12 samples, large $CD21^{+}$ cells accounted for <1%; therefore were classified as negative for MRD. MRD was detected in PB from three dogs, two of which only had PB MRD, whereas the other also showed evidence of MRD in LN and BM, with the latter dog the only one of the study population to demonstrate MRD in BM at this time point.

MRD evaluation by PARR

At T0, the B-cell phenotype of the lymphoma was confirmed in all dogs by PARR, indicating the presence of clonality in IgH gene rearrangements in LN samples. In PB and BM, $PARR^{+}$ results

were found in 10 and 11 dogs, respectively. Ten dogs showed clonal IgH gene rearrangements in both PB and BM, whereas three dogs did not show any evidence for clonal rearrangements in either sample. At T1, $PARR^+$ results were found in 11, 10 and 7 dogs from analysis of LN, PB and BM samples, respectively. Seven dogs were $PARR^+$ in both PB and BM, while four dogs did not show evidence of clonal rearrangements in either sample.

Concordance between FC and PARR for detection of MRD

At T0, the concordance rate between FC and PARR was 100%, 78.6%, and 64.3% for LN, PB and BM, respectively. The highest concordance rate was obtained with LN samples, with all dogs showing $PARR^+/FC^+$ results, whereas the highest discordance was obtained with BM samples, with three dogs being $PARR^+/FC^-$. At T1, samples showed a lower level of consistency, with an agreement rate of 35.7%, 50%, and 57.1% for LN, PB and BM samples, respectively; mostly due to $PARR^+/FC^-$ results being obtained. In contrast, BM samples showed a higher level of agreement, with seven cases being $PARR^-/FC^-$ (Table 1). Overall, 11 dogs had detectable MRD in at least one sample at T1. In 3/11 dogs, both FC and PARR results were positive in at least one sample. In 8/11 dogs, PARR was positive, while FC did not show any detectable malignant cells. In three dogs, MRD could not be detected at T1 by either method in any sample.

Table 1.

Agreement between flow cytometry (FC) and polymerase chain reaction amplification of antigen receptor genes (PARR) for lymph node (LN), peripheral blood (PB) and bone marrow (BM) at T0 and T1 ($n = 14$).

Time point	Sample	$PARR^+/FC^+$	$PARR^-/FC^+$	$PARR^+/FC^-$	$PARR^-/FC^-$
	LN	14	0	0	0
T0	PB	7	0	3	4
	BM	7	1	4	2
	LN	2	0	9	3
T1	PB	3	0	7	4
	BM	1	0	6	7

MRD and clinical outcome

In total, 10 dogs relapsed, either during treatment ($n = 2$) or afterwards ($n = 8$). Nine of these dogs showed $PARR^+$ results in at least one of the samples assessed (Table 2). Conversely, FC was not able to detect MRD in seven of the 10 dogs that relapsed and malignant cells were detected by histopathology in only 2/10 dogs that relapsed. Of the four dogs in CR, both methods gave negative results in all samples for two dogs that had not relapsed, 636 and 602 days from T0, and 436 and 420 days from T1, respectively. The third dog in CR, but with LN $PARR^+/FC^+$ results, died 69 days after T1 for lymphoma-unrelated causes. The LN sample from the fourth dog in CR was $PARR^+/FC^-$, who also died from lymphoma-unrelated causes, 342 days after T0 and 154 days from T1.

Table 2.

Clinical outcome and molecular profiling of 14 dogs with diffuse large B-cell lymphoma (DLBCL).

Dog	Histopathology at T1	FC results at T1	PARR results at T1	TTR (days)	LLS (days)
1	Follicular atrophy	LN^{-}, PB^{+}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	30	237
2	Follicular atrophy	LN^{-}, PB^{+}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	69	530
3	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{-}, PB^{-}, BM^{-}	436[a]	636[a]
4	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{-}, PB^{-}, BM^{-}	420[a]	602[a]
5	Follicular atrophy	LN^{+}, PB^{+}, BM^{+}	LN^{+}, PB^{+}, BM^{+}	8	257
6	DLBCL	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	0	214
7	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	63	569[a]
8	DLBCL	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{-}	0	199
9	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{-}, BM^{-}	154[a]	342
10	Follicular atrophy	LN^{+}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	69[a]	237
11	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{-}	34	272
12	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{-}, PB^{-}, BM^{-}	153	365[a]
13	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{-}	85	364[a]
14	Follicular atrophy	LN^{-}, PB^{-}, BM^{-}	LN^{+}, PB^{+}, BM^{+}	63	383[a]

TTR, time to relapse; LSS, lymphoma-specific survival; LN, lymph node; PB, peripheral blood; BM, bone marrow.

aOngoing.

Correlation between MRD and TTR

PARR was more sensitive than FC in predicting TTR (Table 3). When considering MRD, based on analysis of LN samples at T1, median TTR was 63 days for dogs with $PARR^{+}$ results, and was not reached for dogs with $PARR^{-}$ results ($P = 0.0492$). Conversely, the difference in TTR comparing FC^{+} and FC^{-} dogs was not statistically significant. Dogs that were $PARR^{+}/FC^{-}$ for LN samples had a significantly shorter median TTR, compared with dogs demonstrating $PARR^{-}/FC^{-}$ results ($P = 0.0448$; Fig. 2a). All other combinations were not statistically significant.

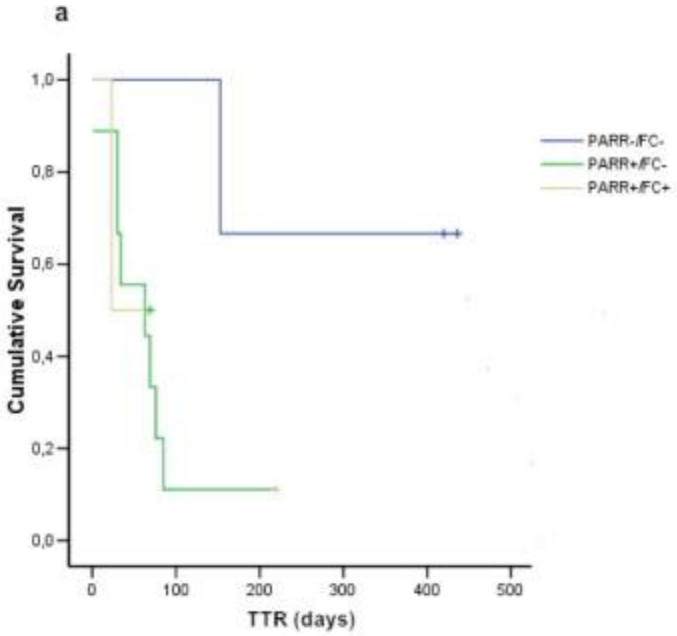
Table 3.

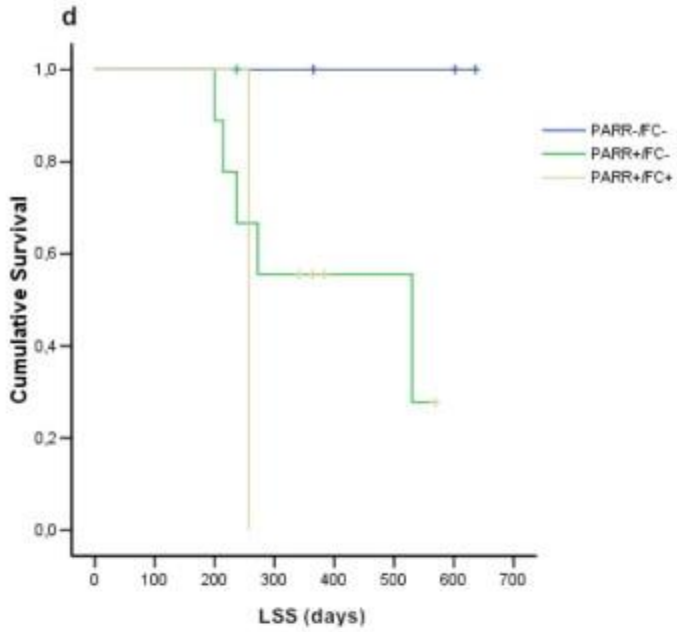
Time to relapse (TTR) and lymphoma-specific survival (LSS) according to FC and PARR results.

Endpoint	Sample	Technique	Median (days)	Mean (days)	95% CI	Log rank test	*P*-value
TTR	LN	$PARR^{+}$	63	66	26–106	3.87	0.0492
		$PARR^{-}$	NA	342	191–493		
		FC^{+}	23	46	14–78	0.02	NS
		FC^{-}	69	152	56–247		
	PB	$PARR^{+}$	34	46	26–66	8.54	0.0035
		$PARR^{-}$	NA	365	245–485		
		FC^{+}	30	41	13–69	3.44	NS

Endpoint	Sample	Technique	Median (days)	Mean (days)	95% CI	Log rank test	*P*-value
LSS	BM	FC^-	85	181	70–292	3.89	0.0487
		$PARR^+$	63	48	26–71		
		$PARR^-$	153	226	87–364		
		FC^+	23	23	23–23	3.01	NS
		FC^-	76	161	64–258		
	LN	$PARR^+$	530	400	330–497	2.75	NS
		$PARR^-$	NA	534	NA		
		FC^+	257	257	257–257	0.4	NS
		FC^-	530	483	377–589		
	PB	$PARR^+$	272	383	281–485	3.75	NS
		$PARR^-$	NA	486	NA		
		FC^+	257	341	156–527	3.10	NS
		FC^-	NA	521	410–632		
	BM	$PARR^+$	530	405	282–528	1.03	NS
		$PARR^-$	NA	522	387–656		
		FC^+	257	257	257–257	1.47	NS
		FC^-	NA	490	388–592		

CI, confidence interval; NA, not applicable; NS, not significant.


a
Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
100
200
300
400
500
TTR (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+


d
Cumulative Survival
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0,8
0,6
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100
200
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400
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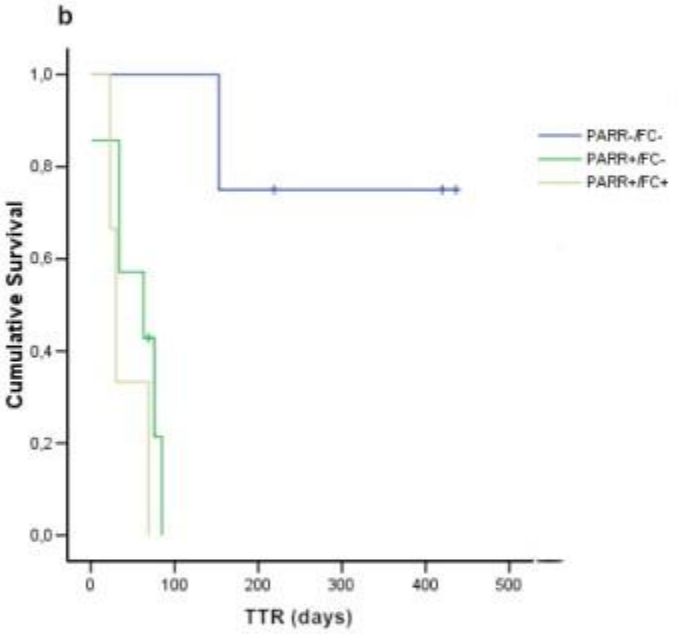
600
700
LSS (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+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Cumulative Survival
1,0
0,8
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0,2
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100
200
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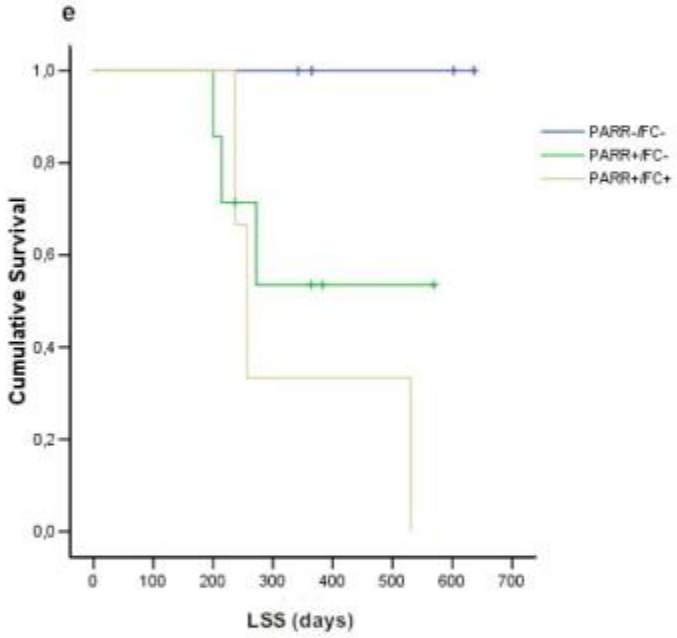
TTR (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+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Cumulative Survival
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0,8
0,6
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0
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300
400
500
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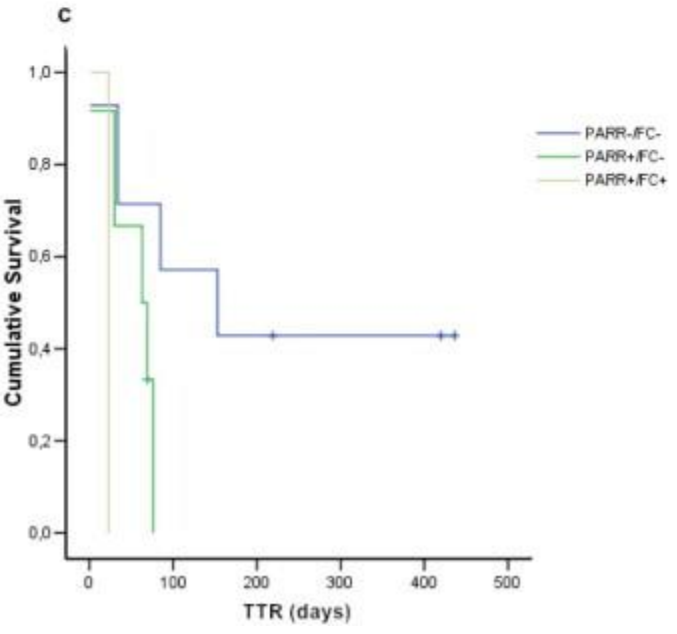
700
LSS (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+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Cumulative Survival
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0,8
0,6
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200
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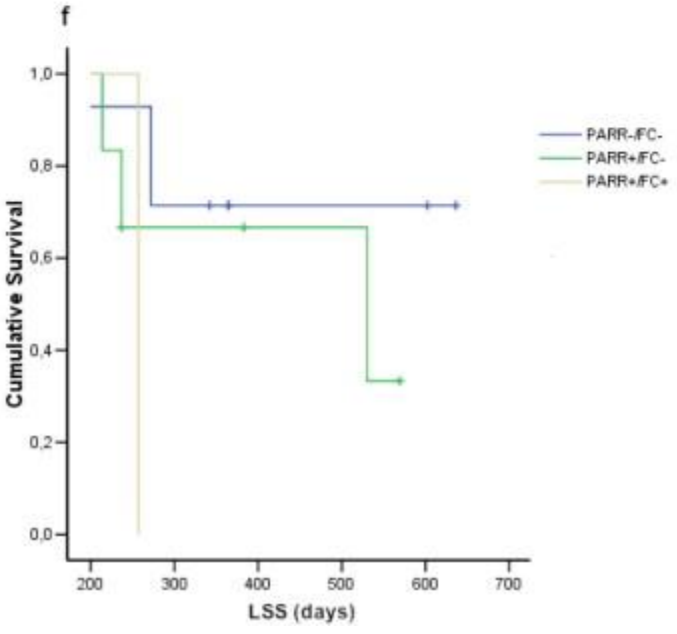
TTR (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+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Cumulative Survival
1,0
0,8
0,6
0,4
0,2
0,0
200
300
400
500
600
700
LSS (days)
PARR-/FC-
PARR+/FC-
PARR+/FC+

Fig. 2.

Kaplan–Meier curve of time to relapse (TTR) for dogs with $PARR^{-}/FC^{-}$, $PARR^{+}/FC^{-}$ and $PARR^{+}/FC^{+}$ results in (a) lymph node, (b) peripheral blood and (c) bone marrow. Kaplan–Meier curve of lymphoma-specific survival (LSS) for dogs with $PARR^{-}/FC^{-}$, $PARR^{+}/FC^{-}$ and $PARR^{+}/FC^{+}$ results in (d) lymph node, (e) peripheral blood and (f) bone marrow. + censored cases.

When evaluating PB samples, median TTR was 34 days for dogs with $PARR^{+}$ results, and was not reached for dogs with $PARR^{-}$ results ($P = 0.0035$). The difference between FC^{+} and FC^{-} samples was not statistically significant. $PARR^{-}/FC^{-}$ PB samples were associated with a longer TTR, compared with those that were $PARR^{+}/FC^{+}$ ($P = 0.001$) or $PARR^{+}/FC^{-}$ ($P = 0.0058$; Fig. 2b).

Dogs with $PARR^{+}$ results for BM samples had a significantly shorter TTR, compared with dogs demonstrating $PARR^{-}$ results (63 and 153 days, respectively; $P = 0.048$). The difference between FC^{+} and FC^{-} dogs was not statistically significant. None of the FC/PARR combinations were statistically significant (Fig. 2c).

Correlation between MRD and LSS

When considering MRD, based on analysis of LN, PB or BM at T1, there was no significant difference in LSS between $PARR^{+}$ and $PARR^{-}$, or between FC^{+} and FC^{-} samples (Fig. 2). Similarly, none of the FC/PARR combinations were statistically significant for LN or BM. For PB samples, a $PARR^{-}/FC^{-}$ result was associated with a longer LSS when compared with $PARR^{+}/FC^{+}$ results (784 and 257 days, respectively; $P = 0.0218$; Fig. 2e) and the combination of PARR and FC was more sensitive than PARR or FC alone in predicting LSS (Table 3).

Discussion

In the present study, we investigated immunological (FC) and molecular (PARR) methods for detection of MRD in samples from 14 dogs affected with DLBCL. Each of these techniques has its own relative advantages, with selection largely dependent upon the expertise and facilities available, and on the purpose for which the method is being used. Advantages of FC include wide applicability, relatively quick results, low cost, capacity to sort residual lymphoma cells, and ability to distinguish between viable and non-viable cells. Advantages of PARR include easily interpretable results and potential for high sensitivity (Gaipa et al., 2012).

The first aim of the study was to compare results obtained either by FC or PARR using the same samples. Overall, our data showed a moderate level of agreement, with results obtained in samples taken at T0, when the tumour burden was relatively high, showing a higher level of agreement, compared with samples taken during follow-up, after initiating treatment. Thus, with decreasing tumour burden in the animal, the sensitivity of FC became more critical, compared with PARR.

At T0, there was full agreement between the two assays when analysing LN samples, whereas the greatest degree of discrepancy was found with BM. Several factors might have influenced this result. Firstly, two different BM aspirates were used for MRD detection by FC or PARR, and it has been demonstrated that the cellularity in a second BM aspiration, taken through the same needle, is likely to be lower, because of haemodilution (Lobetti-Bodoni et al., 2013). Secondly, the limited antibody panel available for canine samples may not allow optimal detection of malignant cells, thus impacting on the sensitivity of FC. At a threshold value of 1%, large B-cells in PB and BM, the

concordance rate between FC and PARR was quite low. It is likely that MRD levels in these cases were <1% and, thus, below the limit of detection for FC.

At T1, the concordance rate for BM and PB was similar to T0, while 9/14 LN samples showed discordant results. Interestingly, in nine dogs, LN analysis by PARR showed evidence of clonality, whereas FC failed to detect MRD. One explanation for this is the possibility of low level infiltration of neoplastic cells in the LN, which is detectable by PARR, but below the limit of detection by FC. Furthermore, in the present study, PARR was performed on tissue samples collected in RNAlater, whereas FC was performed on cell suspensions, collected by fine-needle aspiration, which might not necessarily be representative of the neoplastic infiltration. Alternatively, a variable proportion of neoplastic cells could have undergone apoptosis and would therefore have been excluded from the FC analysis (Gribben et al., 1992), but still likely detectable by PARR. Based on our results, LN molecular staging increased the diagnostic value of histopathological and flow-cytometric staging.

Some of the dogs included in the study had received an autologous vaccine, which might interfere with detection of MRD. In a previously published paper, MRD could be detected, despite administration of chemo-immunotherapy, suggesting that the autologous vaccine did not impede detection of residual neoplastic cells (Marconato et al., 2014).

In humans suffering from lymphoma, monitoring of MRD is currently used for therapeutic stratification and as a measure of outcome in clinical trials (Mitterbauer-Hohendanner et al, 2004, Yang et al, 2005 and Pott et al, 2013). We investigated whether MRD, determined by FC and/or PARR following treatment, had any prognostic impact on TTR and LSS. Although dose-intense chemotherapy leads to CR in the majority of dogs, most will relapse, and frequently die (Garrett et al, 2002, Simon et al, 2006 and Marconato et al, 2011). It is anticipated that monitoring for MRD might be used as a prognostic indicator, identifying those dogs with a high probability of relapse, so that more intense treatment can be initiated, and dogs with a lower probability of relapse can receive more conservative management, to reduce the risk of adverse effects of anti-cancer therapy.

We found significant differences in TTR, when evaluating the two different methods (FC and PARR) and the three different samples (LN, PB and BM). The PARR assay was a better predictor than FC, when considering all analyses. There was no difference in TTR between dogs with FC-detectable and undetectable MRD, regardless of the sample type. Thus, FC is likely to have a poor negative predictive value, with PARR representing a more sensitive method for predicting relapse. When used in combination on PB samples, $PARR^{+}/FC^{+}$ cases had shorter TTR when compared with $PARR^{-}/FC^{-}$ or $PARR^{+}/FC^{-}$ cases.

It is noteworthy that only those dogs with $PARR^{-}/FC^{-}$ results in all three samples remained in CR. In contrast, dogs with $PARR^{+}/FC^{-}$ results eventually relapsed. Also, the absence of detectable MRD in PB and BM by PARR was not sufficient for predicting long-term remission. In terms of LSS, only the absence of MRD by both FC and PARR in PB correlated with a longer survival. However, these data need to be interpreted cautiously, as they could have been influenced by subjective client bias regarding end-of-life decisions.

Conclusions

FC and PARR provide interpretable data on MRD in DLBCL that can be applied for risk assignment. The use of PARR should offset the possibility of false-negative FC results in MRD evaluation. Indeed, if FC does not demonstrate clear evidence of residual disease, this does not imply MRD negativity, and PARR may be required. However, one potential limitation of routine of use of PARR for monitoring of MRD is that the technique is relatively labour-intensive and time-

consuming. On the other hand, if neoplastic cells have been identified by FC, this is suggestive of MRD and PARR is not necessary. When considering all analyses, LN seems to represent the best tissue source to detect MRD by PARR.

As the number of dogs in the current study is relatively small and the follow-up short, conclusions about the clinical application of our research findings await confirmation. Further studies are needed, using FC and PARR, to monitor for MRD, to determine the prognostic value of these methods and their ability to segregate animals into risk categories. It remains to be shown whether earlier detection of an impending relapse, using PARR, is clinically advantageous.

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.

References

References

- Burnett, R.C., Vernau, W., Modiano, J.F., Olver, C.S., Moore, P.F., Avery, A.C., 2003. Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. Veterinary Pathology 40, 32–41.
- Gaipa, G., Cazzaniga, G., Valsecchi, M.G., Panzer-Grümayer, R., Buldini, B., Silvestri, D., Karawajew, L., Maglia, O., Ratei, R., Benetello, A., et al., 2012. Time point-dependent concordance of flow cytometry and real-time quantitative polymerase chain reaction for minimal residual disease detection in childhood acute lymphoblastic leukemia. Haematologica 97, 1582–1593.
- Garrett, L.D., Thamm, D.H., Chun, R., Dudley, R., Vail, D.M., 2002. Evaluation of a 6-month chemotherapy protocol with no maintenance therapy for dogs with lymphoma. Journal of Veterinary Internal Medicine 16, 704–709.
- Gentilini, F., Turba, M.E., Forni, M., 2013. Retrospective monitoring of minimal residual disease using hairpin-shaped clone specific primers in B-cell lymphoma affected dogs. Veterinary Immunology and Immunopathology 153, 279–288.
- Gribben, J.G., Saporito, L., Barber, M., Blake, K.W., Edwards, R.M., Griffin, J.D., Freedman, A.S., Nadler, L.M., 1992. Bone marrows of non-Hodgkin's lymphoma patients with a bcl-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. Blood 80, 1083–1089.
- Lana, S.E., Jackson, T.L., Burnett, R.C., Morley, P.S., Avery, A.C., 2006. Utility of polymerase chain reaction for analysis of antigen receptor rearrangement in staging and predicting prognosis in dogs with lymphoma. Journal of Veterinary Internal Medicine 20, 329–334.
- Lobetti-Bodoni, C., Mantoan, B., Monitillo, L., Genuardi, E., Drandi, D., Barbero, D., Bernocco, E., Boccadoro, M., Ladetto, M., 2013. Clinical implications and prognostic role of minimal residual disease detection in follicular lymphoma. Therapeutic Advances in Hematology 4, 189–198.
- Marconato, L., Stefanello, D., Valenti, P., Bonfanti, U., Comazzi, S., Roccabianca, P., Caniatti, M., Romanelli, G., Massari, F., Zini, E., 2011. Predictors of long-term survival in dogs with high-grade multicentric lymphoma. Journal of the American Veterinary Medical Association 238, 480–485.

- Marconato, L., Martini, V., Aresu, L., Sampaolo, M., Valentini, F., Rinaldi, V., Comazzi, S., 2013. Assessment of bone marrow in filtration diagnosed by flow cytometry in canine large B cell lymphoma: Prognostic significance and proposal of a cut-off value. The Veterinary Journal 197, 776–781.
- Marconato, L., Frayssinet, P., Rouquet, N., Comazzi, S., Leone, F.V., Laganga, P., Rossi, F., Vignoli, M., Pezzoli, L., Aresu, L., 2014. Randomized, placebo-controlled, double-blinded chemo-immunotherapy clinical trial in a pet dog model of diffuse large B-cell lymphoma. Clinical Cancer Research 20, 668–677.
- Martini, V., Melzi, E., Comazzi, S., Gelain, M.E., 2013. Peripheral blood abnormalities and bone marrow in filtration in canine large B-cell lymphoma: Is there a link? Veterinary and Comparative Oncology. doi:10.1111/vco.12024.
- Merli, M., Arcaini, L., Boveri, E., Rattotti, S., Picone, C., Passamonti, F., Tenore, A., Sozzani, L., Lucioni, M., Varettoni, M., et al., 2010. Assessment of bone marrow involvementin non-Hodgkin's lymphomas: Comparison between histology and flow cytometry. European Journal of Haematology 85, 405–415.
- Mitterbauer-Hohendanner, G., Mannhalter, C., Winkler, K., Mitterbauer, M., Skrabs, C., Chott, A., Simonitsch-Klupp, I., Gleiss, A., Lechner, K., Jaeger, U., 2004. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). Leukemia 18, 1102–1107.
- Pott, C., Brüggemann, M., Ritgen, M., van der Velden, V.H.J., van Dongen, J.J.M., Kneba, M., 2013. MRD detection in B-cell non-Hodgkin lymphomas using Ig gene rearrangements and chromosomal translocations as targets for real-time quantitative PCR. Methods in Molecular Biology 971, 175–200.
- Sato, M., Yamazaki, J., Goto-Koshino, Y., Takahashi, M., Fujino, Y., Ohno, K., Tsujimoto, H., 2011. Increase in minimal residual disease in peripheral blood before clinical relapse in dogs with lymphoma that achieved complete remission after chemotherapy. Journal of Veterinary Internal Medicine 25, 292–296.
- Sato, M., Yamzaki, J., Goto-Koshino, Y., Takahashi, M., Fujino, Y., Ohno, K., Tsujimoto, H., 2013. The prognostic significance of minimal residual disease in the early phases of chemotherapy in dogs with high-grade B-cell lymphoma. The Veterinary Journal 195, 319–324.
- Simon, D., Nolte, I., Eberle, N., Abbrederis, N., Killich, M., Hirschberger, J., 2006. Treatment of dogs with lymphoma using a 12-week, maintenance-free combination chemotherapy protocol. Journal of Veterinary Internal Medicine 20, 948–954.
- Thilakaratne, D.N., Mayer, M.N., MacDonald, V.S., Jackson, M.L., Trask, B.R., Kidney, B.A., 2010. Clonality and phenotyping of canine lymphomas before chemotherapy and during remission using polymerase chain reaction (PCR) on lymph node cytologic smears and peripheral blood. The Canadian Veterinary Journal 51, 79–84.
- Vail, D.M., Michels, G.M., Khanna, C., Selting, K.A., London, C.A., 2010. Response evaluation criteria for peripheral nodal lymphoma in dogs (v1.0) – A Veterinary Cooperative Oncology Group (VCOG) consensus document. Veterinary and Comparative Oncology 8, 28–37.
- Valli, V.E., San Myint, M., Barthel, A., Bienzle, D., Caswell, J., Colbatzky, F., Durham, A., Ehrhart, E.J., Johnson, Y., Jones, C., et al., 2011. Classification of canine malignant lymphomas according to the World Health Organization criteria. Veterinary Pathology 48, 198–211.
- Yamazaki, J., Baba, K., Goto-Koshino, Y., Setoguchi-Mukai, A., Fujino, Y., Ohno, K., Tsujimoto, H., 2008. Quantitative assessment of minimal residual disease (MRD) in canine lymphoma by using real-time polymerase chain reaction. Veterinary Immunology and Immunopathology 126, 321–331.

- Yamazaki, J., Takahashi, M., Setoguchi, A., Fujino, Y., Ohno, K., Tsujimoto, H., 2010. Monitoring of minimal residual disease after multidrug chemotherapy and its correlation outcome in dogs with lymphoma: A proof of concept l. Journal of Veterinary Internal Medicine 24, 897–903.
- Yang, M.H., Yen, C.C., Chiang, S.C., Bai, L.Y., Lee, K.D., Hsiao, L.T., Chao, T.C., Wang, W.S., Liu, J.H., Chiou, T.J., et al., 2005. Prognostic significance of clonal diversity of immunoglobulin gene rearrangements in patients with diffuse large B-cell lymphoma. Oncology Reports 13, 503–508.