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Comparison of Free Light Chain assays: Freelite^{TM} and N Latex in diagnosis, monitoring and predicting survival in light chain amyloidosis

Running head
Comparison of Free light chain assays: Freelite^{TM} and N Latex.

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Abstract

Objectives: Measurement of serum free light chains (FLC) is critical in diagnosis, prognosis and monitoring treatment responses in AL amyloidosis. The Freelite^{TM} assay (polyclonal antibodies to hidden light chain epitopes) the current gold standard. We compare a new assay - a mixture of monoclonal antibodies to light chain epitopes (N latex). Methods: 240 serum samples from 94 consecutively newly diagnosed AL amyloidosis patients (at least 3 serial serum samples during the first 6 months) analysed at the National Amyloidosis Centre, London from January 2011 to April 2012. Concordance in detecting abnormal light chain components and haematologic response was assessed at 2, 4 and 6 months. Results: Kappa and lambda clonal light chain involvement; 21% and 79% respectively, with an abnormal kappa/lambda ratio or detectable protein in 78.7%. Median kappa, lambda and difference in involved and uninvolved FLCs by Freelite^{TM} and N-Latex assays: 17.3mg/L versus 16mg/L (R^2=0.91), 48.8mg/L versus 52.6mg/L (R^2=0.52) and 43.2mg/L versus 39.1mg/L respectively. Discordant kappa/lambda ratios at presentation: 10/90 abnormal by Freelite^{TM}; normal by N Latex and 11/90 abnormal by N Latex; normal by Freelite^{TM}. Conclusions: Both FLC assays show good correlation in detecting the abnormal light chain subtype with discordance in absolute values, thus not interchangeable.

Introduction

Systemic AL amyloidosis is a protein deposition disease in which the underlying clonal proliferation of plasma cells produce excess of unstable immunoglobulin light chains, with deposition of immunoglobulin light chains in tissues and organs leading to impairment of the latter.[1, 2] Tracking and monitoring AL amyloidosis is a challenge as most patients have barely a detectable intact monoclonal immunoglobulin. The discovery of serum free light chains as a measure in 2000, led to a development that revolutionised this rare disease. The serum Freelite™ assay measures immunoglobulin free light chains using polyclonal sheep antibodies directed against hidden epitopes on the light chain molecule giving an accurate measurement of serum free kappa and lambda. Our group initially reported the utility of this assay in 2002[3] in this disease, with many studies following. This assay now forms a standard part of the baseline and serial follow up assessments in systemic AL amyloidosis[4, 5], and is part of the revised Mayo staging system and standard international consensus criteria for disease response assessment.[6-8]

Polyclonal antibodies to light chain epitopes in the Freelite™ assay continue to be prepared from specifically immunised sheep. However, in any naturally occurring antibody mixture, standardisation remains complex and inter-assay variability is a distinct possibility. Efforts have been made to develop monoclonal antibodies to the hidden light chain epitopes that would recognise and replicate the success of the polyclonal assay and allow better inter-assay standardisation. A group in the Netherlands working with Siemens, Germany, has developed this technology using a mixture to two kappa and two lambda monoclonal antibodies to the hidden epitopes of the constant region of the immunoglobulin light chain molecules.[9] The references

ranges of both the new N-Latex assay and the polyclonal Freelite^{TM} assay are similar.

With increasing adoption by laboratories of the novel assay, it is important to assess the utility of the new assay compared to the current reference Freelite^{TM} assay. We report a comparison of serum free light chains measured both immunoassays at diagnosis and three further time points in during the initial chemotherapy for patients with systemic AL amyloidosis.

Materials and Methods

This study was conducted at the National Amyloidosis Centre, London, UK (NAC). Consecutive patients with systemic AL amyloidosis seen at the NAC and under prospective follow up from January 2011 and April 2012 undergoing chemotherapy for AL amyloidosis were included, if they had a baseline serum sample at initial diagnosis and at least 3 of 4 blood samples available including from baseline 2, 4 and 6 month follow up point intervals following treatment with chemotherapy. As per standard clinical practice, all newly diagnosed systemic AL patients have monthly serum samples taken during the first year of diagnosis. Diagnosis of amyloidosis was confirmed in all cases with a tissue biopsy demonstrating characteristic birefringence on Congo red staining. Typing of AL amyloidosis was confirmed by immunohistochemical staining with appropriate antibodies and by exclusion of hereditary amyloidosis, where necessary, by genetic sequencing of the genes implicated. All patients underwent systematic review at presentation and detailed follow up assessments at 6 monthly intervals or as clinically indicated. Assessment included clinical examination, detailed blood and urine analysis (including assessment of serum and urine monoclonal immunoglobulin and serum free light

chains at diagnosis and at 6 months), serial ^{123}I labelled serum amyloid P component (SAP) scintigraphy to assess the whole body amyloid load, electrocardiogram (ECG) and an echocardiogram. Organ involvement was defined according to the international amyloidosis consensus criteria.(18) Mayo disease stage was defined as per the criteria published by Dispenzieri et al and Kumar et al.[8,10]

Serum samples were separated and stored at -80ºC. All retrieved samples were tested in duplicate for measurement with Freelite^{TM} (The Binding site Ltd, Birmingham, UK) and N-Latex (Siemens Healthcare Diagnostics, Germany) according to the manufactures' protocols on a BN^{TM}II System nephelometer (Siemens, Germany). The precision of the assay (%CV) was confirmed at multiple light chain concentrations and also within-batch precision. The CV was <5% when analysing a single sample multiple times at all concentrations. The between batch CV (measuring a single sample in one run, then again in a second run, and again in a third run etc) was also <5% at all FLC concentrations except at low very concentrations of <2mg/L the CV is 12.5% for kappa; for lambda values within and between batch %CV is <10% at all levels. Samples are checked for prozone phenomenon by diluting samples where there is a suspicion of prozone using 1:2000 dilution compared to the standard 1:100 dilution. If the result of the 1:2000 dilution is more than 4x different to the 1:100 result then we state that prozone has been detected and amend the result accordingly. {Vercammen, 2011 #6261}. Immunofixation was done using the Sebia Hydrasys analyser using agarose gels (XXX companyXX). Since this is a qualitative method, we run positive and negative controls but cannot assess the precision of it, nor do we do recovery experiments.

Haematologic response was assessed on results of the free light chain (FLC) assays in available samples at 2 months; 90 Freelite^{TM} and 90 N Latex, 4 months; 91

Freelite^{TM} and 89 N Latex, 6 months; 61 Freelite^{TM} and 62 N Latex. Discrepancies in the number of available samples for analysis were secondary to inadequate quantity of serum or error readings in the analysed sample, hence excluded for analysis. In total, serum free light chains were measured in a total of 240 serum samples in duplicate using the Freelite^{TM} and N Latex assays.

We performed a correlation analysis between the 2 FLC assays of results for kappa, lambda and kappa/lambda ratio, examining the qualitative comparison. Haematologic response was assessed as per the international amyloidosis consensus criteria,[6,7] typically developed using results obtained from the Freelite^{TM} assay. This study was not powered to develop new response criteria for the N-latex assay. Statistical analysis was performed using SPSS software with a p value of less than 0.05 considered to be significant. We have stated median values with minimum and maximum ranges. We calculated the Pearson's coefficient to evaluate the agreement and concordance between the two FLC assays, with use of scatter plots to illustrate this. Kaplan Meier curves were used to assess prognostic utility of both assays.

Results

A total of 94 patients were identified from the National Amyloidosis database over this period of time. The available samples at each time point included: at baseline; 90 Freelite^{TM} and 90 N Latex, 2 months; 90 Freelite^{TM} and 90 N Latex, 4 months; 91 Freelite^{TM} and 89 N Latex, 6 months; 61 Freelite^{TM} and 62 N Latex. All patients had a baseline sample as per inclusion criteria. The median age was 64 years (range 55.2-72.2 years) at diagnosis and 48 (51%) male. Cardiac involvement was present in 43%; 23% with Mayo stage 3, renal involvement in 72 (76%), liver involvement in

10 (10%). Of the patients in this group, 74 (78.7%) had either a monoclonal protein detectable in the serum, urine by immunofixation or had an abnormal free light chain ratio, with 20 (21.3%) having no abnormal free light chain ratio or M protein by urine or serum investigations. 50 (53.2%) of this group had a measurable monoclonal protein ≥ 1g/L. The revised Mayo disease stage, which incorporates the dFLC, using FLC measured by Freelite^{TM}/N-Latex assays: Stage 1: 20.2%/25.5%, Stage II: 47.9%/43.6%, Stage III: 29.8%/30.9%, and Stage IV 2.1%/0%, respectively.

The FLC in baseline samples showed an abnormal kappa in 41% and lambda in 63% by Freelite^{TM} assay, and abnormal kappa in 32% and 67% by N Latex assay. The kappa and lambda light chain was 17 (18%) and 75 (82%) respectively. The Freelite^{TM} assay showed a median kappa of 17.3mg/L (range 0.3-1440), median lambda 48.8mg/L (range 7.5-1430) and median difference in involved and uninvolved light chains (dFLC) of 43.2 mg/L (range 55-247.9). The median abnormal kappa was 34.75mg/L (range 0.3-1440) and abnormal lambda 93.85mg/L (range 27-1430). The N latex assay had corresponding concentrations: median kappa 16mg/L (range 2.35-770), median lambda 52.6mg/L (range 3.65-786) and median dFLC 39.1 mg/L (range 58.3-247). The median abnormal kappa by the N-Latex assay was 28.1 (range 2.35-770) and abnormal lambda 77.9 (3.7-786). There was not a significant difference in the absolute kappa and lambda values obtained by either assay (kappa median 34.8 vs. 28.1 $p = 0.64$; lambda median 93.85 vs. 77.9; $p = 0.41$).

The concordance between the two assays was assessed by categorising the results into serum kappa, serum lambda and kappa: lambda ratio as abnormally high, normal and abnormally low as per the normal ranges by each manufacturer. The concordance for the serum kappa light chain was 85% with R^2=0.91 and Pearson

correlation co-efficient r=0.804 (p = 0.0001). The concordance for serum lambda was 75.5% with R^2=0.52 and Pearson correlation coefficient r=0.50 (p=0.0001). The concordance for the kappa/lambda ratio for the respective values 78.8%, R^2=0.87 and r=0.97 (p=0.0001) (Fig 1A, 1B and 1C respectively and Table 1).

There were discordant kappa/lambda ratios at presentation with 10/90 abnormal by Freelite™ but normal by N Latex assay, and 11/90 abnormal by N Latex and normal by Freelite™ (Table 2). There were 10 patients with an abnormal κ/l ratio by Freelite™ assay (normal by N Latex assay): positive IFE by both urine and serum (n=4), positive IFE only by serum (n=2) and positive IFE only by urine (n=3), negative IFE by urine and serum (n=1). There were 11 patients with an abnormal κ/l ratio by N Latex assay (normal by Freelite™ assay): positive IFE by both urine and serum (n=3), positive IFE by only serum (n=3), positive IFE by only serum (n=4) and negative IFE by urine and serum (n=1).

The diagnostic sensitivity (true positive) and specificity (true negative) with agreement of the κ/l ratios of the FreeliteTM and N Latex assays were calculated (Table 3 and Table 4).

We evaluated the clinical impact of these differences in assessing haematologic response according to the revised Consensus criteria[7] of those with measurable dFLC at 2, 4 and 6 months post chemotherapy treatment for patients with sampels available at each of these time points. At baseline 54 (60%) and 51 (56.7%) had a dFLC >50 mg/L (the minimum defined to assess the free light chain response). Using Freelite™ and N Latex assays respectively, at 2 months, a complete response (CR) was achieved in 23% and 32%, partial response (PR) in 17% and 14% and no response (NR) in 57% and 49% of the evaluable patients. There was a discrepancy

in the proportion classed responders at each time point as depicted in fig 2A and 2B. At 2 months, an abnormal kappa/lambda ratio was present in 45.6% by Freelite^{TM} and 46.7% by N Latex assays. Monitoring of the kappa/lambda ratio by both assays produced similar results, although there were subtle differences at 4 months; with 34.4% and 38.2% and 6 month; 30% and 36.1% by Freelite^{TM} and N Latex assays respectively (figure 2).

The estimated overall survival (OS) of the 94 patients was 24.1 months with a median follow-up of 8.6 months. All patients were treated with chemotherapy regimens including: Cyclophosphamide/Thalidomide/Dexamethasone (CTD), n=36, Bortezomib/(Cyclophosphamide)/Dexamethasone (VCD), n=21, Revlimid/Dexamethasone, n=4, Melphalan/Prednisolone/(Thalidomide) (MPT), n=5, Rituximab based regimens n=4, CTD followed by CVD, n=21, MP followed by VD n=3. Given the differences in the proportion of patients achieving a partial response (PR) by both assays at 4 months, we evaluated the OS of those achieving a partial response or greater by both assays. There was a significant improvement in the OS for patients achieving a partial response or better at 4 months by each assay, with the median OS not reached in either case. The survival benefit for those achieving a PR or better by Freelite^{TM} at 4 months was HR 7.84, 95% CI (0.94-65.57), log rank p = 0.03 and by the N-Latex assay HR 95.47, 95% CI (0.11-79601), log rank p = 0.004.

Discussion

We report here the comparative utility of using two different assays (one using polyclonal anti-light chain antibodies and other using a combination of monoclonal anti-light chain antibodies) for detection of and serial monitoring of serum free light

chains in patients with systemic AL amyloidosis undergoing chemotherapy. This study finds that the two assays are broadly comparable for detection of abnormal light chains in the serum with a greater concordance for detection of kappa than for lambda immunoglobulin free light chain. However, discrepancies were also present with the numbers abnormal, the absolute light chain value and time point to reach thresholds for haematological responses different in some which may also impact on survival.

The Freelite™ assay was the only assay available for measurement of serum free light chains until the development of the N-Latex assay, allowing a different method to study these complex molecules; but standardisation is needed between the two assays. Both assays have been studied in myeloma as well as normal controls to assess imprecision, underestimation; antigen excess or non-linear reactivity and overestimation; polymeric forms reacting at multiple antigenic FLC sites of results.[11-13] However, it is important to emphasise that the free light chain component in Myeloma is higher than in patients with AL amyloidosis, and as such direct extrapolation of the results from our study cannot be translated with regard to Myeloma patients. Our study was not focused on the biochemical or technical aspects of either assay, but was designed to assess the clinical utility of each assay in a routine clinical setting in systemic where the serum free light chains are typically abnormal, but low and where FLC measurements form the cornerstone of patient assessment and monitoring. In previous studies in patients with a monoclonal gammopathy, the kappa showed the best correlation between both FLC assays, followed by kappa/lambda ratios and then lambda, with the Pearson coefficients 0.97, 0.83 and 0.91 respectively,[14] and other studies have also shown comparable results.[15, 16] Pretorius et al compared the utility of both Freelite™ and N Latex assays

in 116 samples showing large variations between both assays, precluding the interchange of results, with statistically significant non-linearity occurring in approximately half of the monoclonal and polyclonal samples (kappa and lambda) for both assays.[17]

The current study confirms that in systemic AL amyloidosis, there is, in general, excellent concordance between both the assays in detecting an excess of serum free light chains with better concordance of kappa than lambda as previously reported. The overall concordance in our study for kappa, lambda and kappa/lambda ratios were 85.5%, 75.5% and 78.8% respectively - similar to another study 70-80% [14] but lower than another study quoting the concordance as approximately 90%.[9] The diagnostic sensitivity with respect to the serum and urine IFE results was lower than expected in both assays, 54.3% and 56.7% by FreeliteTM and N Latex assays respectively. Some patients had lower serum free light chain values at diagnosis, with the presence of serum and/or urine IFE, clinical symptoms and diagnostic biopsy needed to prove the diagnosis. The serum free light chain level is often lower than in Myeloma patients. Approximately 10% of cases with each assay detected an abnormal FLC ratio that was not detected by the other assay. The underlying reason for this discordance is difficult to fully explain, given the details of antibodies used to detect the free light chains in both assays are commercially confidential. Due to the nature of the monoclonal disease underlying AL amyloidosis, the mutational signature of the plasma cell DNA in each AL light chain is unique and each light chain sequence is unique in every AL patient. That is most likely explanation leading to the anti-light chains antibodies in each assay do not recognising the epitopes on the respective light chains and hence there is a false negative signal. The Mayo clinic group, using mass spectrometry, has recently demonstrated that such samples

also contain a monoclonal light chain. This suggests that currently neither method is perfect for detection of all abnormal monoclonal free light chains but as different patients “detected” or “missed” by either assay, there may be a role for repeating the FLC measurement by the other assay if an abnormality is not detected in a patient with one assay. This may help to, at least partly, overcome the problem in AL amyloidosis of lack of evaluable light chains in a certain proportion of all patients.[3] We compared the κ/l ratios and the IFE results showing that both assays had similar sensitivities (54.3 and 56.7%) and specificities (86.2 and 86.8%) for the FreeliteTM and N Latex assays respectively.

This is the first study to examine serial parallel samples to assess the serum free light chain responses by both assays simultaneously. Although there is broad agreement in the response assessment as a percentage of total patients with both assays, there were important differences in the proportion of patients who reach the current threshold used to define a partial or very good partial response. Eighteen patients reached a PR and VGPR earlier by N-Latex assay than the same class of response by the Freelite™ assay. This is likely as the assays have very different antibodies which measure the free light chain in serum and hence the actual slopes of the standard curves are not same. The response criteria in AL amyloidosis have been derived using ROC analysis based on survival of patients using various light chain thresholds obtained by the Freelite™ assay. Thus the current thresholds are clinical and not biochemical end points. The N-Latex assay classified patients as responders at an earlier time point than the Freelite™ assay, hence designing a study to examine a clinically relevant threshold for defining a response with N-latex is important. Mollee et al described a comparison of these 2 assays in 62 patients with 32 samples post treatment also showing variations in the dFLC response and hence

haematologic response. The median reduction in dFLC was 68% for the N-Latex assay and 77% for the Freelite™ assay (p=0.04), with a partial response assigned by both assays predicting overall survival (N-Latex p=0.0015, Freelite™ p=0.022). {Mollee, 2013 #6262}

Achieving a haematologic response translates into a survival benefit. However, the earlier classification of a greater proportion of patients as responders by the N-Latex assay has risks, with the treatment duration in amyloidosis often shorter than in myeloma, with a lower threshold to stop treatment earlier in the responders given the toxicity and tolerance to the treatments. Unless thresholds are redefined, patients classed as responders by the N-Latex assay may get undertreated and hence carry a risk of shorter time to disease progression.

This current study needs to be interpreted in the context of certain limitations. The numbers are small and all patients did not have serial samples available. Although the patients recruited in this study are serial AL patients seen at the NAC, there is a selection bias as all patient were required to have serial samples for six months i.e. only six month survivors are recruited thereby excluding a substantial number of patients with stage III cardiac AL amyloidosis where there is still 30-40% mortality in the first six months.[18] A study of truly unselected serial patients is needed to assess the true utility of these assays. There was higher than expected proportion of patients with non-evaluable light chains, likely due to selection of the survivors who are typically patients with early stage disease and lower serum free light chain burden.

In conclusion, both Freelite™ and N Latex assays are important in detecting the abnormal FLC in patients with systemic light chain amyloidosis. Our study

demonstrated excellent correlation between the assays for detecting the abnormal light chain subtype, with discordance in the absolute values of the FLC suggesting that cross assay interpolation is impossible. Furthermore, each assay appears to have limitations, in missing random yet different patients suggesting a need by both manufacturers to further optimize the anti-light chain antibodies underpinning the respective assays. Studies using clinical end points are needed to harmonize the response assessment criteria for each assay; given the values may not be equivalent and validation of the new assay is important in clinical and trial results if this emerges as a more widely used assay in diagnosing and monitoring systemic AL patients. Designing future clinical trials in AL amyloidosis incorporating FLC measurement by both assays would make a substantial contribution to our understanding and harmonisation of the field.

Author contributions

SM, and ADW designed the study and wrote the manuscript. NW performed the laboratory analysis and SJS provided laboratory biochemistry input. SM and ADW performed the statistical analysis and critically reviewed the manuscript. All the authors SM, NW, SJS, SS, TL, DF, CJW, HJL, JDG, PNH and ADW confirm they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting and revising the article; and (c) final approval of the published article.

Authors' Disclosures or potential Conflicts of interest

No authors declared any conflicts of interests

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Table 1 Concordance analysis for kappa (A), lambda (B) and kappa/lambda ratio (C) for the 2 FLC assays.

Group	Number	N Latex FLC (mg/L)	Freelite^{TM} FLC (mg/L)	Pearson's correlation
FLC κ	90	16.1 (2.35-770)	17.3 (0.3-1440)	0.804
FLC l	90	52.6 (3.65-786)	48.8 (7.5-1430)	0.493
κ/l ratio	90	0.68 (0.01-63.56)	0.88 (0.0002-224.7)	0.97

FLC – Free light chain; mg/L – milligrams per litre; κ – kappa; l – lambda

Table 2. Kappa/Lambda ratios at presentation

		N Latex Ratio		
		Normal	**Abnormal**	**Total**
Freelite™ Ratio	**Normal**	27	11	38
	Abnormal	10	42	52
	Total	37	53	90

Table 3. Clinical sensitivity and specificity of the Freelite^{TM} assay and immunofixation electrophoresis (IFE).

	IFE (serum and urine)		
Freelite^{TM} κ/l ratio	Positive	Negative	Total
Abnormal	44	7	51
Normal	37	2	39
Total	81	9	90

Sensitivity 54.3%, Specificity 86.2%

Table 4. Clinical sensitivity and specificity of the Freelite^{TM} assay and immunofixation electrophoresis (IFE).

	IFE (serum and urine)		
N Latex κ/l ratio	Positive	Negative	Total
Abnormal	46	7	53
Normal	35	2	37
Total	81	9	90

Sensitivity 56.7%, Specificity 86.8%

Figure 1. Scatter plots of N Latex and Freelite^{TM} free light chain assays for (A) kappa N Latex (n=91) and Freelite^{TM} (n=91) and (B) lambda N latex (n=90) and Freelite^{TM} (n=90) and (C) kappa/lambda ratio (n=90) illustrating the concordance of both free light chain assays.

Figure 2A and B. Difference in involved and uninvolved free light chain (dFLC) response in patients with evaluable disease. (A) Percentage of evaluable patients assessed at 2, 4 and 6 months according to haematologic response including no response (NR), partial response (PR), very good partial response (VGPR) and complete response (CR) with (B) illustrated the differences of the abnormal kappa/lambda ratio at set intervals between the Freelite^{TM} and N Latex assay. There was no statistical difference between the 2 assays by chi-square test, illustrated in both figures with corresponding p values.