

RESEARCH ARTICLE

Robust Reference Intervals for Serum Kappa and Lambda Free Light Chains from a Multi Centre Study Population from Hyderabad, India: Myeloma Diagnostic Implications

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Abstract

The International Myeloma Working Group considers the serum free light chain (SFLC) assay to be an adjunct to traditional tests. Apart from the FLC ratio, the absolute values of individual free light chains also are gaining importance as they appear to be more relevant in certain clinical settings. Automated assays are available for their determination. As laboratories put new test systems into use catering to different disease populations, they are required by accreditation and certification bodies to verify or establish performance specifications, including reference intervals (RIs) representative of their population. Our aim was to establish local RIs for SFLC in a multicentre representative healthy population using a robust method. There was no significant relationship between SFLC levels and age, gender and creatinine levels. The 95% RI for κ SFLC was 4.81 to 33.86mg/L, for λ SFLC was 5.19 to 23.67mg/L and for κ/λ SFLC was 0.36 to 2.33, significantly higher than the values given by the manufacturer. The κ/λ SFLC ratio at 2.23, covering 100% of the data, showed 72% sensitivity (95% CI=39.0 - 94.0), 100% specificity (95% CI=71.5 - 100.0), 100% PPV (95% CI=21.5 - 100.0), 95% NPV (95% CI=75.4 - 99.9), and 79% accuracy (95% CI=56.0 - 93.0). In the patient group, kit RI for κ/λ SFLC ratio classified 45.5% (n=5) as positive vs 9.1% (n=1) positive by the study RI, while the kit RI for kappa FLC classified 90.9% (n=10) as positive vs 54.5% (n=6), indicating increased probability of false positive test results with the kit RI when applied to our patient population. Appropriate and specific reference intervals and criteria values result in fewer false-positive and false-negative results which means fewer wrong or missed diagnoses.

Keywords: Robust reference intervals - serum - free kappa light chains - free lambda light chains - myeloma

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Introduction

Monoclonal free light chains (FLCs) are important disease biomarkers in patients with plasma cell-proliferative disorders. The increasing evidence for clonal diversity and evolution in multiple myeloma highlights the importance of laboratory algorithms that measure both intact immunoglobulins and monoclonal FLCs, at diagnosis and when monitoring response to treatment (Jenner 2014). The reference interval for polyclonal FLCs was documented, and the serum reference interval for the free light chain kappa to lambda (κ/λ FLC) ratio was demonstrated to be a sensitive indicator for excess (e.g., clonal) FLC production (Katzmann, Clark et al., 2002). The International Myeloma Working Group (IMWG) considers the serum FLC (SFLC) assay to be an adjunct to

traditional tests (Rajkumar et al., 2011). The assay allows for quantitative monitoring of response and remission after treatment and provides prognostic information (Katzmann et al., 2006; SV. 2011) potentially reducing the need for frequent bone marrow biopsies. The SFLC assay may also be the only means of detecting a disease marker in some disease settings: (1) non secretory MM (NSMM), in which SFLCs are often the only marker of the disease (Mead et al., 2004). (2) AL amyloidosis in which low M protein concentrations may not be detected by means of conventional techniques; and (3) light chain MM (LCMM), in which the M protein consists only of FLCs (SV. 2011). Thus, in addition to detecting a wider spectrum of plasma cell dyscrasias (PCDs) than traditional tests, the assay may help detect earlier stages of the disease, and because of the short half-life of SFLCs (2

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to 6 hours, vs. 21 days for complete immunoglobulins) (Sanchorawala et al., 2005) the assay may also help detect relapses and treatment failures earlier than by reliance on M protein concentrations alone (SV. 2011). Apart from the ratio, the absolute values of individual free light chains also are gaining importance as they are included in the prognostication and appear to be more relevant than the FLC ratio in certain clinical setting (Dispenzieri et al., 2005). This indicates that the SFLC assay is a natural addition to serum and urine IFE for diagnostic testing in the monoclonal gammopathies. Automated assays are available for their determination. Method validation is a standard process in every good laboratory to judge the acceptability of a new method. It is also a requirement of ISO 151895 whenever a particular interval is thought to be no longer appropriate, or where a pre-examination or examination (analytical) procedure is changed (Antony, 2008). Clinicians compare the values laboratory professionals report with the given reference intervals. Whether a test is normal or abnormal is perhaps the most important element of a laboratory test. Most laboratories use reference ranges published in manufacturers' package inserts, even though they are supposed to establish their own. As laboratories put new test systems into use catering to different disease populations, they are required by accreditation and certification bodies to verify or establish performance specifications, including reference ranges.

In the Robust method, by using robust measures of location (center) and scale (spread), the method does not make any assumptions about the underlying distribution of the data. The method involves somewhat more expertise in computing, but the iterations required can be done in typical spreadsheet programs like Microsoft Excel. The exact number of observations required varies.

Aim of the study is to establish local Reference intervals (RIs) for κ & λ free light chains in the serum (κ & λ SFLCs) of representative healthy population by Robust method (Horn et al., 1998; Horn et al., 2005; 2008).

Materials and Methods

This is a multicentre study involving three tertiary care hospitals located in Hyderabad, Telangana, India. (Pool data from many individual sites in order to achieve adequate numbers of samples) (Gary 2008). Participating institutes are Nizam's Institute of Medical Sciences (NIMS), Basavataraka Indo- American cancer hospital & Research Institute (BIACH & RI) and Krishna Institute of Medical Sciences (KIMS). Total number of subjects were 72 with 28 Females and 44 Males. The individuals were selected from a reference population using specific criteria (Clerk et al., 1987), which includes individuals aged between 30 - 75 years without renal failure, congestive heart disease, chronic respiratory diseases, liver diseases, malabsorption syndromes and nutritional anemias, not on any of pharmacologically active agents - alcohol, and tobacco, oral contraceptives, replacement or supplementation therapy e. g. Insulin, without any Systemic diseases - hypertension, diabetes, without any Modified physiological states - pregnancy, psychological and mental disorders such as severe stress

and depression, exercise or physical training, food intake prior to blood collection. The pre-analytical, analytical and post-analytical phases of the analyses were controlled throughout the study (Solberg and PetitClerc 1988). Moreover, a pilot study was conducted before the actual data collection. Care was taken to identify the selection criteria that were reflective of the healthy individuals the lab caters to. Physicians at each center screened and identified persons fulfilling the selection criteria. Sampling was done by a Priori method (2000). Blood was obtained from a total of 72 healthy subjects from three study centers in plain and EDTA tubes. The following blood tests were performed in the serum: Total protein, Albumin, Cystatin C, Creatinine, immunoglobulin A, G & M, Serum protein electrophoresis, kappa FLC and lambda FLC. Using the kappa and lambda FLC results, a FLC ratio was determined.

22 patients referred to us for myeloma work up were included to analyze the RIs given by the study. Written informed consent was obtained from the reference individuals. Institutional ethical committee approved the study.

Method of estimation for Serum Total Proteins, Albumin, and Creatinine was Biuret, BCG & Jaffe rate Blank colorimetric assay respectively. Serum Cystatin C, Serum Immunoglobulin A, G, M & Serum Free Light Chain Kappa, Lambda was estimated by immunoturbidimetry & Agarose gel was used for SPE. Automated analyzers were used at all the three centers. For the analysis of FLCs, Cobas 6000 of Roche Company was used at NIMS, AU series of Beckmann Company was used at BIACH & RI and KIMS. Dedicated reagents were used for routine biochemical parameters and binding site kit is used for analyzing free light chains. The participating centers have strict quality assurance guidelines for all the study parameters.

Analytical performance of the Methods

In the validation stage of methods for Total protein (gm/dl), Albumin (gm/dl), Cystatin C (mg/L) & Creatinine (mg/dl) earlier, control materials demonstrated an intra-assay coefficient of variation (CV %) of 0.8 and 0.7, 0.9 and 0.7, 0.8 & 0.7 and 0.8 & 0.9 at concentrations (mg/L) of 6.0 & 4.5, 3.0 & 2.5, 1.0 & 2.0 and 2.0 & 4.0 respectively. Inter-assay coefficients of variation (CV %) were 1.3 & 2.4, 1.7 & 2.4, 1.2 & 2.1, 3.5 & 3.2 respectively. LJ charts were then plotted and all the quality control results were in the acceptable limits.

We ran 20 replicate tests on polyclonal sera with typical FLC values to determine the CV of the assay. Intra-assay CV % for low & high kappa and lambda light chains is 2.6 & 1.4 and 4.2 & 1.2 respectively at concentrations (mg/L) of 17.71 & 36.20 and 10.0 & 24.50. Inter-assay CV % of kappa free light chains low & high and lambda free light chains low & high is 5.83 & 6.28 and 7.55 & 4.12 respectively at concentrations (mg/L) of 16.4 & 34.0 and 26.1 & 55.0 (Table 1).

Statistical analysis

The data obtained from the analyzer was entered in Microsoft excel sheet and analyzed by MedCalc

Statistical Software version 15.8(2015). Reed et al test and D'Agostino-Pearson (DAP) test (Reed, Henry et al., 1971) were used to detect outlier values and to determine normality of distributions of analyte values respectively. Descriptive statistics was done for all the parameters. Independent samples t test was used to check the mean difference of parameters between males and females. Statistics for Correlation & multiple regressions was applied to the data to see the relationship between SFLC levels and age and between SFLC levels and Creatinine & Cystatin C levels respectively. Partial correlation was used to see the relationship between FLCs and Creatinine & Cystatin C so that partial correlation coefficient is adjusted or corrected for the influence of age. ANOVA is used to compare the groups.

Test of homogeneity of variances gave a p value >0.05 indicating the samples were from a homogenous group.

Comparison of means (t-test) of κ SFLC, λ SFLC and κ/λ FLC ratio between our values and the manufacturers values were done to check the significance of difference if any. Then, the upper and lower end points covering 95% of the reference values, double-sided of the kappa and lambda FLC were determined with their respective 90% confidence intervals (CIs) by using the Robust method, according to the IFCC/CLSI recommendation (Horn, Pesce et al., 1998). Chi-squared test was done to see the classification of the patient subjects by the RIs and diagnostic range given by the kit and our study.

RI calculations for κ & λ free light chains and κ/λ FLC ratio

A robust, CLSI/ IFCC recommended (CLSI, 2008), method (Horn, Lesce et al., 2005) was used for the determination of upper and lower end points covering 95% of the reference values of each analyte with respective 90% CIs using MedCalc Statistical Software version 15.8 (2015). For the robust method the confidence intervals are estimated with the bootstrap method (Efron 1993). A robust Reference Interval estimator determines a Reference Interval from a data sample as small as 20 skewed data samples, even in the presence of outlier samples. This ability avoids expensive tests to increase the data sample or allows calculation of a Reference Interval when only a small cohort for sampling is available. First, a set of data samples are power transformed to remove a non-Gaussian skew to the set. Then, the Reed et al. approach is used to identify an outlier cutoff and the set of data samples are truncated to remove outlier data samples that are beyond the outlier cutoff. The truncated set of data samples are then used to compute the Reference Interval. The truncated sets of data samples are then also power transformed to compute the Reference Interval.

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were estimated for the κ/λ FLC on the basis of both the central 95% interval and a diagnostic range that captured 100% of the test data. Accuracy was calculated as the proportion of individuals classified correctly. Confidence intervals were calculated according to the exact binomial distribution for sensitivity, specificity, and accuracy and by bootstrap for PPV and NPV. We calculated both PPV

and NPV after assuming a 15% prevalence of monoclonal proteins, in the 22 samples submitted to our lab for monoclonal protein studies.

Results

Distribution of the data

We visually inspected the distribution of the data and the values in the tails of the distribution to identify data errors and outliers for each parameter. Serum Lambda free light chain, Total protein, Cystatin C, Creatinine & Globulins had normal distribution. Parametric statistics were used as the data was large and results expressed as mean, median, SD and 95% CI (Table 2). No significant gender differences were seen in the mean, SD & CI for serum concentrations of kappa, lambda FLC & κ/λ ratio, age, globulins & Cystatin C. Two samples with outliers detected for kappa FLC by the method of Reed et al (Reed et al., 1971) were excluded from analysis. We selected the age group between 30 to 75years based on our patient data (unpublished) in the past three years.

There was no relationship between SFLC and age. Independent samples t test showed significant difference in Creatinine (mg/dl) (mean \pm SD=0.99, \pm 0.14, vs 0.75 \pm .16, p=0.00), Total protein (gm/dl) (M=7.5 \pm 0.4 vs F=7.3 \pm 0.3) and Albumin (gm/dl) (mean \pm SD=4.44 \pm .37 vs 4.6 \pm .33, p=0.00) levels between male and females respectively. No significant difference was seen in SFLC levels between males and females. Partial correlation did not show any relationship between SFLC and Creatinine & Cystatin C when adjusted or corrected for the influence age.

An age-related (age-specific or age-dependent) reference interval is a reference interval that varies with the patients' age. The methodology that MedCalc uses to model a continuous age-related reference interval is based on the methods described by Altman (G. 1993) (Altman and Chitty 1993) and (Wright and Royston 1997). The z-scores did not display any pattern and were randomly scattered about 0 at all ages (Altman and Chitty, 1993). And less than 5% of cases lie above

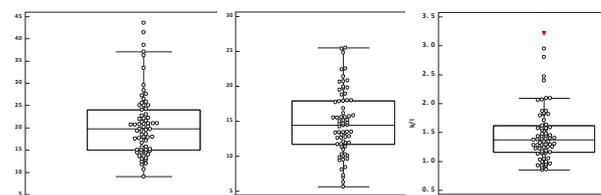


Figure 1. All Data Plots for Kappa SFLC, Lambda SFLC and SFLC Ratio

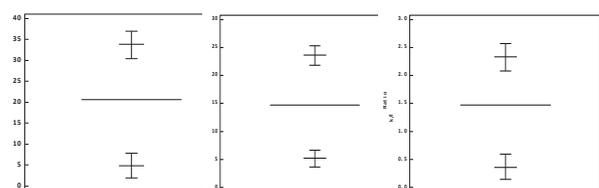


Figure 2. Robust Reference Ranges for Kappa SFLC, Lambda SFLC and SFLC Ratio

the line corresponding to $z=1.645$ and 5% of cases were below the line corresponding with $z=-1.645$; and these cases were randomly distributed across the observed age range. As the age was increasing κ FLCs levels were also increasing but there was no significance when adjusted for Creatinine values. No such relationship was seen with λ FLCs levels and age but with Creatinine levels the values decreased mildly. κ/λ ratio showed slight but not significant narrowing as the age increased.

Reference Intervals

The median for κ SFLC, λ SFLC & κ/λ ratio is 19.8 mg/L, 14.4 mg/L & 1.38 respectively and they were not significantly related to age, sex and Creatinine. 95% Reference interval, Double-sided, showed significantly higher levels of κ FLCs than λ FLCs (4.8-33.9 vs 3.3-19.4, $P=0.001$).

Significantly higher mean levels of κ FLCs (20.7mg/L, difference=12.3, 95%CI=10.93-13.67, $p=0.0001$), λ FLCs (14.7mg/L, difference=1.3, 95%CI=0.31-2.29, $p=0.01$) and κ / λ SFLC (1.47, difference=0.84, 95% CI = 0.761-0.919, $p=0.0001$) were seen as compared to that given by the manufacturer.

The 95% Reference Intervals for κ FLCs is 4.81 to 33.86, for λ FLCs is 5.19mg/L to 23.67mg/L and κ/λ FLCs

ratio is 0.36 to 2.33. These are significantly higher than that reported by the manufacturer ($p<0.001$) (Fig 2, Table 3).

Diagnostic characteristics

As shown in Figure 3, κ/λ SFLC ratio at 2.23, covering 100% of the data, showed 72% sensitivity (95% CI=39.0

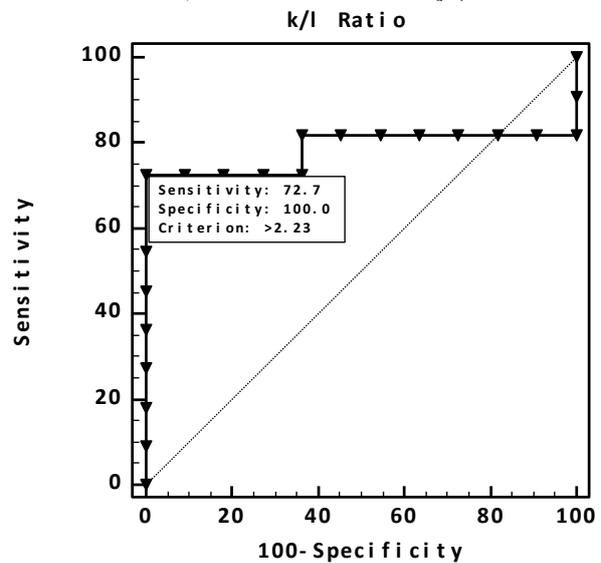


Figure 3. ROC for Kappa and Lambda SFLC Ratio

Table 1. SFLCs Performance Data from the 3 Institutes Analyzers

Source	SFLC	Intra assay (mg/L)	Intra assay CV%	Inter assay (mg/L)	Inter assay CV%
Binding Site Kit	SFLC Kappa	5.58	1.4	5.79	7.2
	SFLC Lambda	18.47	2.5	19.76	7
NIMS	SFLC Kappa	7.66	4.1	7.98	10
		27.34	5.5	28.58	3.6
	SFLC Lambda	17.71	2.5	16.4	5.83
		36.2	1.37	34	6.28
KIMS	SFLC Kappa	10	4.17	26.1	7.55
		24.5	1.18	55	4.12
	SFLC Lambda	8.9	2.5	16.9	6.9
		30.2	1.9	31.9	7.6
BIACH&RI	SFLC Kappa	9.8	4	28	6.5
		28.08	3.9	56.5	8.2
	SFLC Lambda	15.5	2.8	16.98	7.6
		35	1.8	31.95	9.1
		8.8	3.9	28.08	7.12
		31.23	4.12	56.46	8.23

Table 2. Summary Statistics

Variable	Age (yrs)	kappa SFLC (mg/L)	lambda SFLC (mg/L)	k/λ sFLC	Creatinine (mg/dl)	Total Proteins (gm/dl)	Albumin (gm/dl)	Globulins (gm/dl)	Cyst.C (mg/L)
N	70	70	70	70	70	70	70	70	70
Mean	41	20.7	14.7	1.47	0.9	7.4	4.5	2.91	0.96
SD	9	7.2	4.6	0.48	0.2	0.4	0.4	0.38	0.15
Median	39	19.8	14.4	1.38	0.9	7.5	4.6	2.9	0.96
25 - 75 P	33 to 47	15.0 to 24.1	11.7 to 17.9	1.16 to 1.62	0.8 to 1.0	7.2 to 7.7	4.4 to 4.8	2.60 to 3.10	0.90 to 1.05

Table 3. Robust Method (CLSI C28-A3) Reference Intervals, Double-sided

	Kappa SFLC (mg/L)	Lambda SFLC (mg/L)	κ / λ SFLC	
			Reference Intervals(mg/L)	Diagnostic Range(mg/L)
Lower limit	4.81	5.19	0.4	0.36
90% CIa	1.96 to 7.80	3.68 to 6.63	0.14 to 0.60	0.14 to 0.60
Upper limit	33.86	23.67	2.3	2.33
90% CIa	30.43 to 36.94	21.83 to 25.33	2.08 to 2.58	2.08 to 2.58

a Bootstrap confidence interval (10000 iterations; random number seed: 978)

- 94.0), 100% specificity (95% CI=71.5 - 100.0) and with 15% prevalence of the disease it gives PPV 100% (95% CI=21.5 - 100.0), NPV 95% (95% CI=75.4 - 99.9), and accuracy 79%(95% CI=56.0 - 93.0).

Kit κ/λ FLC ratio classified 45.5% (n=5) as positive vs study 9.1% (n=1). Kit kappa FLC classified 90.9% (n=10) as positive vs study 54.5% (n=6).

Discussion

The mean κ SFLC, λ SFLC levels and κ/λ SFLC ratio in our subjects is found to be higher than that reported in the literature. Consequently, reference intervals for S κ FLC and κ/λ SFLC ratio also are higher and that of λ SFLC is lower when compared to that of manufacturers' (Katzmann, Clark et al., 2002). Our subjects higher RI of κ/λ SFLC ratio may reduce false positives in our reference population, thereby enabling the clinician to avoid unnecessary work up of the cases. This will not only reduce the psychological stress on the patient and their family but also help in reducing the financial burden on the patient. The Freelite, Binding site has quoted the RIs published by Katzmann et al. which were established in an American, predominantly white, population. The 95% Reference Intervals of our study against that of the kit are for κ SFLC (mg/L) is 4.81 to 33.86 vs 3.3 - 19.4, for λ SFLC (mg/L) is 5.19 to 23.67 vs 5.7 - 26.3 and κ/λ SFLC ratio is 0.36 to 2.33 vs 0.26-1.65. Known ethnic differences in the normal ranges of total immunoglobulins (Jolliff, Cost et al., 1982) have prompted a number of laboratories to determine normal ranges for their local populations. European research groups (Callis et al., 2008, Hernandez et al., 2010) have reported Freelite reference ranges for normal individuals that are comparable to Katzmann et al. Similarly, SFLC concentrations in a small South African cohort including Black (57/113), mixed-race (44/113) and Caucasian (12/113) subjects were not significantly different, both for the local population as a whole, and for the Black and mixed-race populations independently (Zemlin et al., 2013). In a Han Chinese population of 326 subjects, although a narrower κ/λ SFLC ratio normal range was observed (0.32 - 1.52), this local range and the Katzmann range (0.26 - 1.65) provided the same diagnostic sensitivity and specificity for multiple myeloma (MM) (area under ROC curve 0.99 in both cases) (Liang et al., 2014).

The higher levels of κ free light chains in our study population have important clinical relevance. Firstly, (RI of κ SFLC is 4.81 to 33.86mg/L) helps to report higher proportion of normal. Approximately 40% more light chains than heavy chains are synthesized (Waldmann, Strober et al., 1972) normally, and this excess of FLCs is thought to favor accurate assembly of intact immunoglobulin molecules. Light chains which remain unbound from their heavy chain partner are secreted into the blood as FLCs. Secretion of FLC is highest from plasma cells, with twice as many producing κ -chains than λ -chains. κ FLCs are normally monomeric, while λ FLCs tend to be dimeric, joined by disulphide bonds; however, higher polymeric forms of both FLCs may occur. The normal plasma cell content of the bone

marrow is about 1%, whereas in multiple myeloma (MM) this can rise to over 90%. In chronic infections and autoimmune diseases the bone marrow may contain 5 - 10% plasma cells, and may be associated with hypergammaglobulinaemia and corresponding increases in polyclonal serum FLC (SFLC) concentrations. Our study subjects were thoroughly screened for all these conditions in the exclusion criteria. Serum concentrations of FLCs and intact immunoglobulins reflect the balance between their production and kidney clearance rates. Our subjects had normal S. Creatinine and Cystatin C levels, with no infection and normal SPE. They were as healthy as healthy could be. At around 25 kDa in size, monomeric FLCs, characteristically κ , are cleared in 2 - 4 hours at 40% of the glomerular filtration rate. Dimeric FLCs of around 50 kDa, typically λ , are cleared in 3 - 6 hours at 20% of the glomerular filtration rate, while larger polymers are cleared more slowly (Miettinen and Kekki, 1967; Waldmann et al., 1972). Although κ FLC production rates are estimated to be twice that of λ , their faster removal ensures that actual serum concentrations are approximately 50% lower. Studies in 'healthy' Indians have found a lower range of normal glomerular filtration rates (GFRs) than in Western populations, but it is not clear if this difference is a reflection of subclinical kidney disease or is simply physiological (Mahajan et al., 2005; Barai et al., 2008). In an audit reported by Marshall et al., 4.9% (47/955) of individuals tested had a borderline abnormal κ/λ SFLC ratio (between 1.67 and 3.2) with no known plasma cell disorder (Marshall et al., 2009).

Age and sex did not have any influence on the levels of the analytes similar to that reported in the literature (Katzmann et al., 2002). The apparent association of age and levels of serum Creatinine with the levels of κ SFLC, λ SFLC and κ/λ SFLC did not show statistical significance and the association disappeared on adjustment with covariates. Hence, there was no requirement for different RIs for age and sex.

Interpretation of borderline SFLC assay results at diagnosis or follow up require careful interpretation by the physician which again would vary subjectively necessitating the need of considering additional clinical and laboratory parameters. In the patient group in our study, kit RI for κ/λ FLC ratio classified 45.5% (n=5) as positive vs 9.1% (n=1) by the study RI, kit RI for kappa FLC classified 90.9% (n=10) as positive vs 54.5% (n=6) by the study RI, indicating increased probability of false positive test results by the kit RI when applied to our patient population. The four cases had their kappa, lambda FLCs and ratio values between 27.7 to 46.2 mg/L, 14.5 to 20.3mg/L and 1.88 to 2.23 respectively which were on the borderline. The 100% specificity (95% CI=71.5 - 100.0) of the κ/λ SFLC 2.23 ratio from our study clearly reduces the probability of false positive results.

In conclusion, from our study, we have made 3 novel observations regarding the serum levels of SFLC. First, the serum levels of SFLCs are higher in our population and therefore appropriate for reporting the test values. Secondly, in the light of the increasing use of absolute levels of SFLC levels in the evolving algorithms in the diagnosis and management of various plasma cell

dyscrasias, not only the ratio is important but also the individual absolute values. And finally, establishing appropriate reference intervals for our patient population gives confidence in the information we provide to practicing physicians. Better reference intervals result in fewer false-positive and false-negative results, this means fewer wrong or missed diagnoses.

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