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ORIGINAL ARTICLE

Analytical Performance and Diagnostic Potential of Immunoassays Determining Intact Immunoglobulin κ/λ Ratios in Monoclonal Gammopathies

JACQUELINE ECKOLD [1], WOLFRAM POENISCH [2], TIM DROGIES [1], JUERGEN KRATZSCH [1], DANIEL TEUPSER [1, 3], JOACHIM THIERY [1], MATHIAS BRUEGEL [1, 3]

[1] *Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University Hospital Leipzig, Germany*
[2] *Department of Hematology/Oncology, University of Leipzig, Leipzig, Germany*
[3] *Institute of Laboratory Medicine, Ludwig-Maximilians-University, Munich, Germany*

SUMMARY

Background: **Hevylite™ chain (HLC) assays with specificity for epitopes at the junction between heavy and light chains of intact immunoglobulins (Ig) allow quantification of Ig κ/λ ratios of the three major Ig classes. Calculated Ig κ/λ ratios outside the reference range indicate a monoclonal background. The primary aim of the present study was to analytically validate HLC assays and to investigate their diagnostic potential in relation to immunofixation electrophoresis (IFE) as the standard method for identification of monoclonal proteins (MPs). A second aim was to investigate the diagnostic potential of HLC assays in disease monitoring.**

Methods: **Precision, linearity, accuracy, sensitivity, and specificity of HLC assays for Ig classes A, G, and M were determined as parameters of analytical performance. The diagnostic performance of HLC assays in the detection of MPs was investigated in patient sera revealing monoclonal bands in IFE (n = 156). The utility of the assays in disease monitoring was investigated in a proof of principal approach by quantification of HLC ratios in subsequent sera from stem cell transplanted (ScTx) myeloma patients (n = 4).**

Results: **All six HLC assays revealed analytical performances suitable for application in routine diagnostics. With regard to diagnostic performance, all samples with IgA MPs in IFE (n = 54) could be identified in the HLC IgA assay. Of sera showing IgG MP in IFE (n = 69), 57 could be identified in the HLC IgG assay, whereas 12 had normal IgG κ/λ ratios. Of sera showing IgM MP in IFE (n = 26), 25 could be identified in the HLC IgM assay, 1 serum revealed a normal IgM κ/λ ratio. ScTx patients achieving IFE-negative remission had normal HLC ratios. Those who failed to achieve IFE-negative remission showed normalization of conventional monitoring parameters but revealed HLC ratios never reaching reference range.**

Conclusions: **HLC assays exhibit analytical performances suitable for clinical routine application. Our preliminary data from ScTx patients suggest a diagnostic potential especially of HLC IgA assay in disease monitoring. Other than that, combined application of HLC assays does not represent an alternative to IFE in first line diagnostics, in particular due to the limited diagnostic performance of the HLC IgG assay.**

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KEY WORDS

Hevylite™ chain assays, immunoglobulin κ/λ ratios, monoclonal gammopathy, validation, test performance

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INTRODUCTION

Determination of monoclonal proteins (MPs) in serum is an integral part in screening and monitoring of monoclonal gammopathies (MG) [1]. Currently used detection methods are mainly based on electrophoretic techniques, such as serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE) [2]. Moreover, an

immunoassay measuring free light chains (FLC) has been introduced [3]. Nephelometric or turbidimetric assays are used for quantification of secreted whole immunoglobulins (Ig) [2].

Considering these methods, SPE allows relative quantification of MPs and is therefore primarily used for disease monitoring. Its application in screening is limited by insufficient sensitivity and inability to identify monoclonal Ig class. IFE is characterized by a combination of electrophoretic separation and immunoprecipitation and, due to its sensitivity, currently represents the standard indicator for presence of MPs [4]. However, IFE does not permit quantification of MPs and, thus, has the disadvantage of limited utility in disease monitoring. Another common disadvantage of electrophoretic techniques is the laborious working process and the observer dependent evaluation. Due to a lack of electrophoretic methods for the proper quantification of MPs, nephelometric or turbidimetric methods detecting total Ig concentration are used for disease monitoring. However, these methods do also not accurately reflect the real amounts of MPs [4]. On the other hand, serum FLC assays allow excellent detection of FLCs but are unable to detect monoclonal whole Ig, thus always requiring combination with electrophoretic methods [5,6].

Therefore, an automated immunoassay combining sensitive detection and specific quantification of monoclonal whole Ig potentially allowing reduced application of laborious and observer dependent electrophoretic techniques in diagnosis and monitoring of MGs would be of great interest. Recently, immunoassays targeting unique epitopes between heavy- and light chain constant regions of intact Ig have been introduced (IgA κ and λ, IgG κ and λ, IgM κ and λ Hevylite™ chain (HLC) assay) [7]. These assays allow quantification of serum heavy and light chain pairs for the investigated Ig classes. The calculated HLC ratio allows identification of a monoclonal background.

The primary aim of the present study was to analytically validate all six HLC assays in a clinical setting and to investigate their capability in the detection of MPs in comparison to IFE as the standard test. Furthermore, in a proof of principal approach we investigated the diagnostic potential of these assays in monitoring the course of myeloma in stem cell transplanted (ScTx) patients.

MATERIALS AND METHODS

Study population, serum collection and storage

A total of 119 serum samples from 43 patients with MG type IgA (intact immunoglobulin multiple myeloma (IIMM), n = 39; monoclonal gammopathy of undetermined significance (MGUS), n = 4), 162 serum samples from 59 patients with MG type IgG (IIMM, n = 52; MGUS, n = 7), 32 serum samples from 21 patients with MG type IgM (IIMM, n = 7; Morbus Waldenstrom (MW), n = 5; MGUS, n = 9), and 7 serum samples from patients with light chain multiple myeloma (LCMM) were used to determine the analytical and diagnostic performance of HLC assays. More than one serum sample was selected from different patients in case of multiple sample entry in the course of disease and marked decrease or increase of total Ig concentration. In addition, consecutive serum samples from ScTx IIMM patients (n = 4) were used for assessing the diagnostic potential of HLC assays in disease monitoring. All patients were routinely treated at the University Hospital of Leipzig and received bone marrow diagnostics for classification of malignancy or MGUS. Respective sera were characterized by monoclonal bands in IFE. Sera from patients without laboratory signs of MG but presence of rheumatoid factors (RFs) (n = 10) were used for interference studies. Sera from healthy blood donors (n = 22) were used as controls. All blood samples were centrifuged within 1 hour after blood drawing and serum was stored in aliquots at -20°C until analysis. The study met the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee of the Medical Faculty of the University Leipzig, Germany (082-10-19042010).

Laboratory diagnostics

HLC assays (Hevylite™ human kits for IgA κ, IgA λ, IgG κ, IgG λ, IgM κ, IgM λ), FLC assays (Freelite™ kit) (The Binding Site, Birmingham, England) and determinations of total IgA, IgG, IgM were performed by nephelometric analysis (BNII, Siemens Diagnostics, Eschborn, Germany) according to manufacturer´s instructions. Ratios of HLC and FLC assays were classified according to suggestions of the manufacturer. RFs were analyzed on a BN ProSpec (Siemens Diagnostics, Eschborn, Germany), total protein was analyzed on a clinical chemistry analyzer (Modular, Roche Diagnostics, Basel, Switzerland). IFE and SPE were performed on a Hydrasis electrophoretic system (Sebia, Evry, France). Presence of monoclonal bands in IFE or SPE was assessed by two independent experienced observers.

Phoresis software (Sebia, Evry, France) was used for discrimination of monoclonal peaks in SPE which were semi-quantitatively analyzed in relation to total protein.

Analytical validation of Hevylite™ chain assays

Patient sera containing MPs of isotype IgA κ, IgA λ, IgG κ, IgG λ, IgM κ or IgM λ were mixed into pool sera (four for each isotype), each in appropriate amounts, to form a set of samples covering broad concentration range of the six different HLC assays for determination of intra- and interassay precision. The isotype of these sera was identified by IFE; the Ig concentration was determined by nephelometric analysis. Linearity testing of HLC assays was performed with patient pool sera (n = 2 for each isotype) and then serially diluted into different calibration ranges of several assays. Recovery of HLC assays was investigated by analysis of pooled serum from three healthy subjects spiked with different calibrators. As accuracy criteria, summed concentrations of

Ig κ/λ pairs were determined in 106 sera from 31 patients with MG type IgA, in 156 sera from 53 patients with MG type IgG, and in 28 sera from 18 patients with MG type IgM and were then compared with total Ig concentrations of the respective classes as determined by nephelometry.

To investigate a potential cross-reactivity of different classes of MPs in HLC assays, sera containing MPs type IgG or IgM (n = 5 for each isotype) were analyzed with the HLC IgA assay, sera containing MPs type IgA or IgM (n = 5 for each isotype) were analyzed with the HLC IgG assay, and sera containing MPs type IgA or IgG (n = 5 for each isotype) were analyzed with the HLC IgM assay. To investigate a potential interference of RFs in HLC assays, patient sera with different concentrations of RFs (n = 10) but without monoclonal bands in IFE were analyzed. Functional sensitivity of HLC assays was assessed by 6-fold determinations of patient pool sera containing MPs after dilution up to the limit of detection using 0.1% albumin solution. A comparison of sensitivity between IFE and HLC assays was performed by recurrent analyzes of sera (n = 2 for each Ig class) that were stepwise diluted with 0.1% albumin solution until absence of monoclonal bands in IFE or reaching the limit of detection in HLC assays.

Assessment of diagnostic potential of Hevylite™ chain assays

The capability of HLC assays to detect MPs was investigated by comparative analysis of sera revealing monoclonal bands in IFE (n = 156) as the standard method. To investigate the potential of HLC assays in disease monitoring in a proof of principal approach, sera from ScTx IIMM patients (n = 4) were subsequently collected over a time period of 1½ years and retrospectively analyzed. Results of the HLC assays were compared to IFE as the conventional marker of residual disease and to total Ig concentrations and semi-quantitatively evaluated monoclonal peaks in SPE as quantitative parameters in monoclonal disease.

RESULTS

Analytical performance of Hevylite™ chain assays

Intra- and interassay precision ranged between 1.5% and 19% for HLC IgA assays, 0.8% and 16% for HLC IgG assays, and 1.0% and 8.5% for HLC IgM assays over the calibration range (Online supplement, Table 1). Dilution experiments revealed linearity in the range of 0.80 to 38.8 g/L for HLC IgA assays, 3.40 to 48.5 g/L for HLC IgG assays and 0.80 to 95.0 g/L for HLC IgM κ assay. Due to the fact that patient samples containing MP type IgM λ were not available, we were unable to investigate precision and linearity of the HLC IgM λ assay. Spiking of test calibrators into sera from healthy subjects resulted in a recovery rate ranging from 92 to 108% for HLC assays. Comparison between summed concentrations of IgA κ/λ, IgG κ/λ, and IgM κ/λ pairs and the respective total Ig concentrations revealed an excellent linear correlation with correlation coefficients of 0.97, 0.96, and 0.99, respectively (Figure 1A - C).

We next tested cross-reactivity by testing HLC assays for IgA with MP type IgG and IgM, HLC assays for IgG with MP type IgA and IgM, and HLC assays for IgM with MP type IgA and IgG. Maximum concentration of MP type IgA, IgG or IgM in sera used for cross-reactivity testing was 36.0 g/L, 46.0 g/L, or 102 g/L. As shown by normal IgA κ/λ ratios, MPs type IgG or IgM did not cross-react in the HLC IgA assay until the highest serum concentrations (Figure 2A). As shown by normal IgG κ/λ ratios, MPs type IgA did not cross-react in the HLC IgG assay until maximum serum concentration of 36.0 g/L. However, patient sera exclusively containing MP type IgM above a serum concentration of 16.0 g/L revealed HLC IgG κ/λ ratios outside the reference range (Figure 2B). Cross-reactivity persisted after dilution of these sera. Furthermore, these sera revealed differences between total IgG concentrations and summed concentrations of IgG κ/λ pairs. MPs type IgA or IgG did not cross-react in the HLC IgM assay up to the highest serum concentrations (Figure 2C). In some cases, IgM κ/λ ratios were found marginally outside the reference value; however, agreement of summed concentrations of IgM κ/λ pairs with total IgM concentrations argues against cross-reactivity (data not shown). In addition, potential analytical interferences of HLC assays with RFs were analyzed in serum samples containing RFs in concentrations from 20.0 to 600 U/mL. HLC IgA, IgG and IgM assays were not affected up to the highest concentrations as confirmed by HLC Ig κ/λ ratios in reference ranges (Figure 2A-C).

6-fold determination of diluted pool sera revealed a functional sensitivity of 0.02 g/L for IgA κ/λ assay, 0.13 or 0.05 g/L for IgG κ or IgG λ assay, and 0.01 for IgM κ/λ assay (data not shown). Sensitivity of HLC assays was tested by serial dilution of 2 representative sera for each class of MPs up to 1:32 (Figure 3A-F). In addition, detectability of MPs in diluted sera was tested by IFE. A recovery of MPs using the HLC IgA κ assay (Figure 3A) and IgA λ assay (Figure 3B) between 80 and 120% could be shown up to a 1:16 or 1:8 and 1:32 or 1:16 dilution for investigated sera, corresponding to serum concentrations of IgA κ and IgA λ MPs in the range of 0.05 to 0.1 g/L. IFE revealed monoclonal bands up to a 1:32 dilution, corresponding to serum concentrations of IgA κ and IgA λ in the range of 0.02 g/L. The HLC IgG κ assay (Figure 3C) and IgG λ assay (Figure 3D) revealed recoveries between 80 and 120% of MPs up to a 1:4 or 1:8 and 1:16 dilution, corresponding to serum concentrations of IgA κ and IgA λ MPs in the range of 0.1 g/L. IFE revealed monoclonal bands up to a 1:16 or 1:32 dilution for different sera, corresponding to serum concentration of IgG κ and IgG λ MPs between 0.04 and 0.05 g/L. A 80 to 120% recovery of MPs in the HLC IgM κ assay could be shown up to a 1:16 dilution (Figure 3E) and up to a 1:8 or 1:16 dilution in the HLC IgM λ assay, corresponding to serum concentrations of

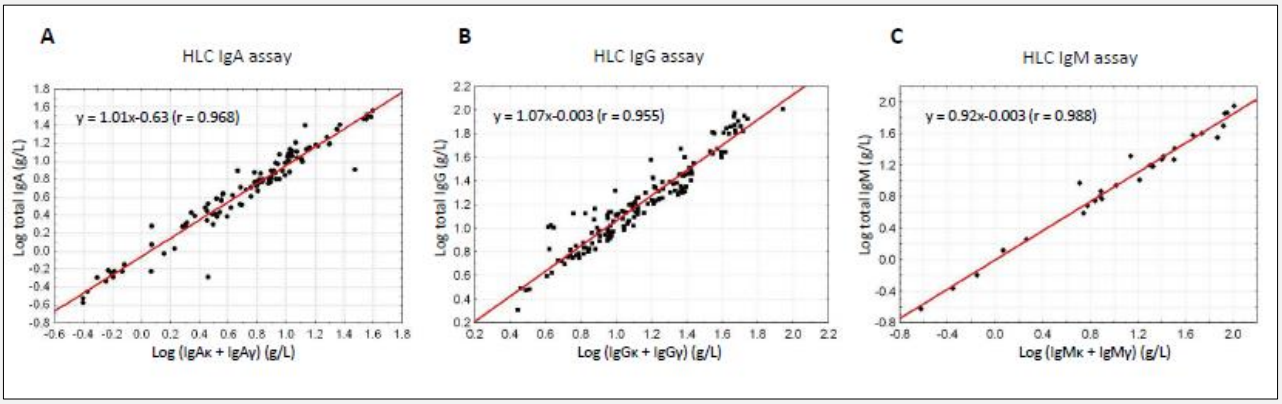


Figure 1. Correlations of summed concentrations of Ig κ/λ pairs and total Ig concentrations.

Patient sera containing monoclonal protein type (A) IgA (n = 106), (B) IgG (n = 156) or (C) IgM (n = 28) were analyzed. Summed concentration of Ig κ/λ pairs detected with HLC IgA κ/λ, IgG κ/λ or IgM κ/λ assay (x-axis) and total Ig concentration determined with nephelometry (y-axis) are shown on a logarithmic scale.

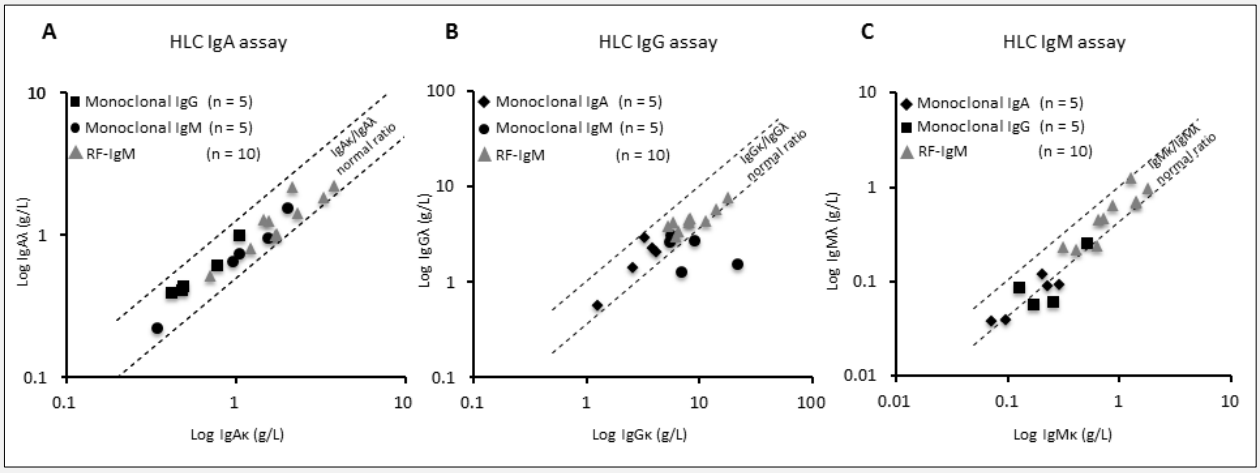


Figure 2. Cross-reactivity of monoclonal proteins and rheumatoid factors in Hevylite™ chain assays.

(A) Patient sera containing monoclonal protein type IgG (dark squares, n = 5) or IgM (dark circles, n = 5) in IFE were analyzed with the HLC IgA assay. (B) Patient sera containing monoclonal protein type IgA (dark rhombus, n = 5) or IgM (dark circles, n = 5) in IFE were analyzed with the HLC IgG assay. (C) Patient sera containing monoclonal protein type IgA (dark rhombus, n = 5) or IgG (dark squares, n = 5) in IFE were analyzed with the HLC IgM assay. Patient sera containing rheumatoid factors (grey triangles, n = 10) were analyzed with the HLC IgA, IgG, and IgM assay (A-C). Concentrations of Ig κ (x-axis) or λ (y-axis) isotypes are shown on a logarithmic scale, dotted lines indicate normal ranges of Ig κ/λ ratio.

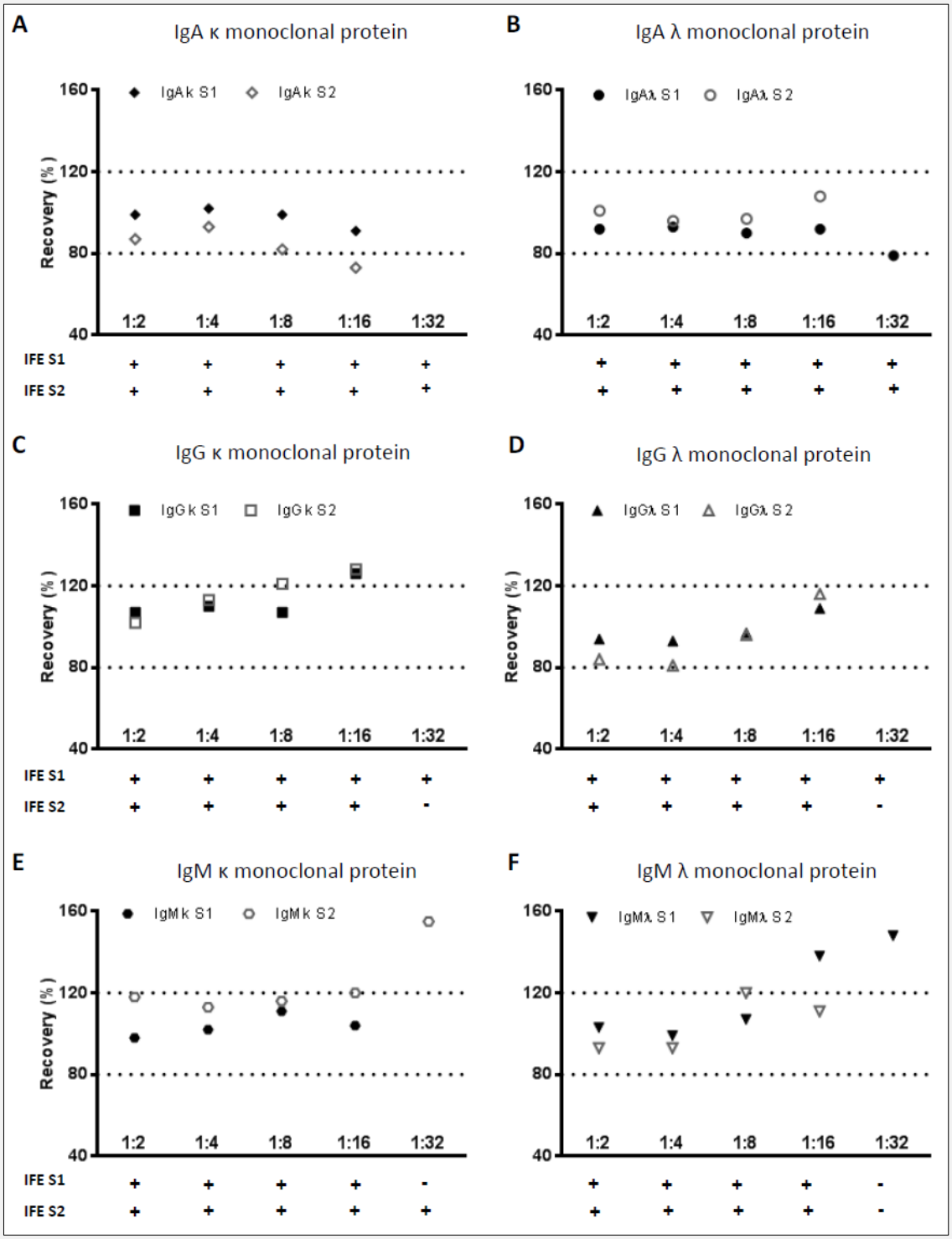
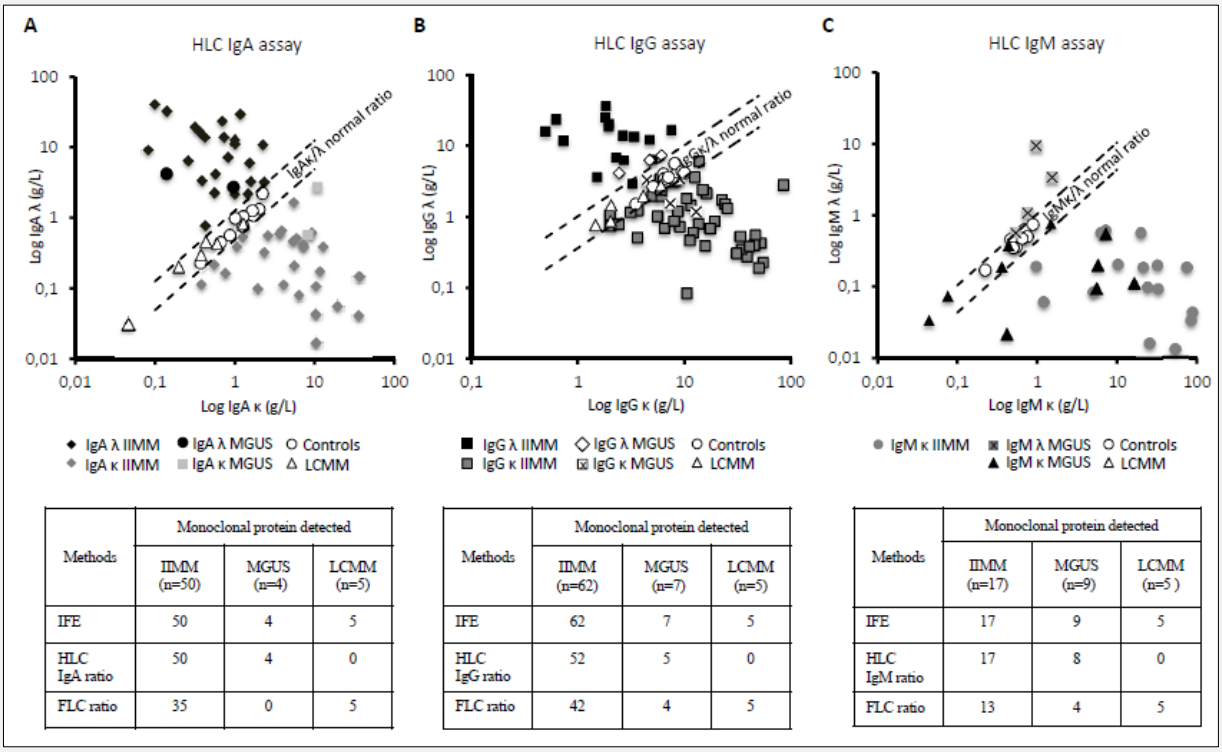


Figure 3. Recovery and sensitivity testing of Hevylite™ chain assays compared to immunofixation electrophoresis after step-wise dilution.

Sera from patients with MG type IgA κ or λ, IgG κ or λ, IgM κ or λ (A-F) were used for sensitivity testing (n = 2 for each isotype). Sera were analyzed at baseline with HLC assays and IFE and subsequently after stepwise dilution until reaching the limit of detection in HLC analysis or absence of monoclonal bands in IFE. Ig κ or λ concentrations and the respective Ig κ/λ ratios are presented as percent recovery relative to baseline. Dotted lines indicate a 20% deviation band. Results of IFE are described as "+" with monoclonal band or as "-" without monoclonal band. IFE, immunofixation electrophoresis; S1, serum 1; S2, serum 2.



Methods	Monoclonal protein detected		
	IIMM (n=50)	MGUS (n=4)	LCMM (n=5)
IFE	50	4	5
HLC IgA ratio	50	4	0
FLC ratio	35	0	5

Methods	Monoclonal protein detected		
	IIMM (n=62)	MGUS (n=7)	LCMM (n=5)
IFE	62	7	5
HLC IgG ratio	52	5	0
FLC ratio	42	4	5

Methods	Monoclonal protein detected		
	IIMM (n=17)	MGUS (n=9)	LCMM (n=5)
IFE	17	9	5
HLC IgM ratio	17	8	0
FLC ratio	13	4	5

Figure 4. Diagnostic potential of Hevylite™ chain assays in the detection of monoclonal protein in comparison to immunofixation electrophoresis.

Sera from IIMM or MGUS patients containing monoclonal protein type (A) IgA (n = 54; black or grey rhombus for IIMM subtype λ or κ, black circles or light grey squares for MGUS subtype λ or κ), (B) IgG (n = 69; black or dark grey squares for IIMM subtype λ or κ, white rhombus or crossed squares for MGUS subtype λ or κ) or (C) IgM (n = 26; grey circles for IIMM subtype κ, grey crossed squares or black triangles for MGUS subtype λ or κ) in IFE were analyzed with the corresponding HLC assays. Sera from LCMM patients (n = 5, white triangles) and healthy controls (n = 10, white circles) were analyzed with HLC assays. Concentrations of κ (x-axis) or λ (y-axis) isotypes are shown on a logarithmic scale, dotted lines indicate normal ranges of Ig κ/λ ratio.

IgM κ and IgM λ MPs in the range of 0.05 to 1.0 g/L. IFE revealed monoclonal bands at least up to a 1:16 dilution, corresponding to serum concentrations of IgM κ and IgM λ between 0.03 and 0.06 g/L.

Comparison of Hevylite™ chain assays and immunofixation electrophoresis in the detection of monoclonal proteins

In total, 156 sera revealing monoclonal bands in IFE (MG type IgA, n = 54; MG type IgG, n = 69; MG type IgM, n = 26; MG type LC, n = 7), and 22 sera of healthy subjects without laboratory evidence for monoclonal gammopathy were analyzed with HLC assays. As indicated by IgA κ/λ ratios outside the reference range, monoclonality could be identified by HLC IgA assays in all investigated serum samples (IIMM, n = 50; MGUS, n = 4) (Figure 4A). As indicated by IgG κ/λ ratios outside the reference range, monoclonality could be identified by HLC IgG assays in 57 sera from IIMM patients. However, 12 sera revealing monoclonal bands in IFE had normal HLC IgG κ/λ ratios (IIMM, n = 10; MGUS, n = 2) (Figure 4B). As indicated by IgM κ/λ ratios outside the reference range, monoclonality could be identified by HLC IgM assays in 25 sera from myeloma (immunocytoma, n = 10; Waldenstrom macroglobulinemia, n = 7), and MGUS patients (n = 8). One serum of a patient with MGUS revealing a monoclonal band in IFE had a normal HLC IgM κ/λ ratio (Figure 3C). All these patients had IgM κ MPs and because no suitable patient samples were available, the diagnostic sensitivity of the HLC IgM λ assay could not be investigated. Sera from healthy subjects and from patients with LCMM (n = 7) revealed normal Ig κ/λ ratios in HLC analysis (Figure 4A-C).

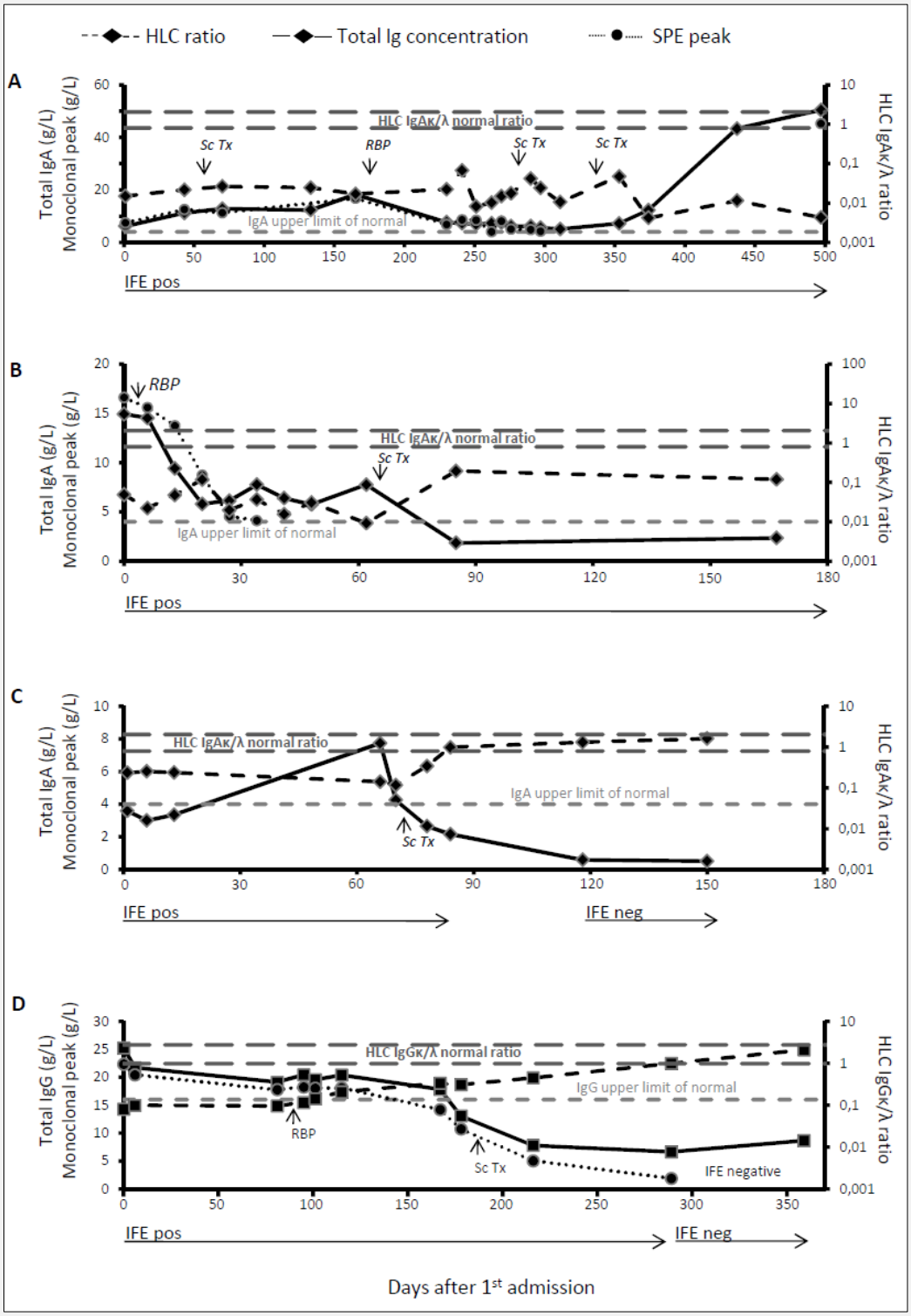


Figure 5. Diagnostic potential of Hevylite™ chain assays in disease monitoring.

HLC analysis was performed on consecutive sera from ScTx patients suffering from IIMM type (A-C) IgA λ (n = 3) or (D) type IgG λ (n = 1). HLC ratios (broken line), total Ig concentrations (solid line), monoclonal peak in serum protein electrophoresis (dotted line) are shown. Dark grey broken lines indicate normal ranges of immunoglobulin κ/λ ratio, light grey broken line indicate upper limit of total Ig concentration. HLC, Hevylite™ chain; IFE, immunofixation electrophoresis; ScTx, stem cell transplantation; RBP, Revlimid, Bendamustine, Prednisone; VMP, Velcade, Melphalan, Prednisone.

Potential of Hevylite™ chain assays in disease monitoring

In a proof of principal approach, intact Ig κ/λ pairs were determined in sequentially collected sera from four ScTx IIMM patients (P1 - P3: Type IgA λ; P4: Type IgG λ). The chronological sequence of results from HLC analysis, total Ig analysis, SPE, and IFE for the four study patients are presented in Figure 5A - D. As shown in Figure 5A, P1 revealed a parallel trend of total IgA concentrations, semi-quantitatively determined monoclonal peaks in SPE and HLC IgA κ/λ ratios up to day 170. Interventions were followed by disappearance of monoclonal peak in SPE and normalization of IgA levels. In accordance with IFE revealing persistent monoclonal bands, HLC IgA κ/λ ratios showed up- and downturns but never reached the reference range. Disease progression after day 350 was associated with recurrence of monoclonal bands in SPE, increase of total IgA, and decrease of HLC IgA κ/λ ratio. As shown in Figure 5B, P2 revealed a parallel trend between total IgA concentrations and HLC IgA κ/λ ratios up to day 70, the monoclonal peak in SPE had already disappeared around day 35. ScTx was followed by a persistent decrease of total IgA concentrations. In accordance with IFE revealing persistent monoclonal bands indicating incomplete remission, HLC IgA κ/λ ratios increased but without reaching the reference range. As shown in Figure 5C and 5D, ScTx in P3 (IIMM type IgA λ) and P4 (IIMM type IgG λ) were followed by a persistent decrease of total Ig concentrations. In accordance with IFE revealing the disappearance of monoclonal bands and indicating complete remission, HLC Ig κ/λ ratios reached reference ranges.

DISCUSSION

The present study demonstrated the analytical quality characteristics of HLC assays suitable for application in routine diagnostics. A high concordance between HLC IgA and IgM assays to IFE in the detection of MPs was shown. However, primarily based on the limited diagnostic sensitivity of the HLC IgG assay, combination of HLC assays will not allow reduced application of IFE as the standard indicator for the presence of MPs. In addition, HLC IgD and IgE assays, which would be required for MP screening are not available, yet. Our preliminary studies performing HLC analysis in ScTx IIMM patients suggest a diagnostic potential, especially of the HLC IgA assay in disease monitoring.

To the best of our knowledge, our study is the first to comprehensively investigate analytical quality parameters and diagnostic performance of all presently available HLC assays in a clinical setting. Based on the idea that application of HLC assays might allow reduced application of laborious and observer dependent electrophoretic techniques, the diagnostic performance in the detection of MPs was investigated in particular in comparison to IFE as the current standard method. An intraassay precision below 12% was shown for the six HLC assays (Online supplement, Table 1), which was in agreement with recent data published by Bradwell et al. [7]. These authors described intraassay precisions of the HLC IgA and IgG assays between 1.5 and 7.4%; the assessment of the HLC IgM intraassay precision was not performed. HLC assays revealed interassay precisions ranging from 1.1 to 8.0% in the concentration range up to 0.56 g/L. Interassay precisions up to 19% were shown for HLC IgA and IgG assays in the lower concentration ranges. Bradwell et al. showed interassay precisions between 1.50 and 7.40% over all concentration ranges; the assessment of HLC IgG and IgM interassay precision was not performed [7]. The analytical performance of HLC assays is further supported by our data showing adequate accuracy in linearity and recovery testing.

Nephelometric analysis of Igs is performed for the quantification of polyclonal or monoclonal proteins indicating bone marrow suppression or tumour burden in gammopathy diagnostics [8,9]. The correlation of HLC IgA, IgG, and IgM assays with nephelometric analysis of polyclonal proteins and of the HLC IgA assay with nephelometric analysis of MPs has been previously demonstrated [7,10]. In the present study we confirmed the correlation of the HLC IgA assay with the nephelometric analysis of MPs but extended these results to HLC IgG and IgM assays by showing a correlation with nephelometric analysis of IgG and IgM MPs in patient sera (Figure 1). Whether HLC assays more accurately reflect Ig secretion as compared to nephelometric analysis of total Igs sometimes overestimating notably IgM levels will have to be investigated in subsequent studies.

In a further step, we were not able to show interferences with RFs as a common disturbing factor in the immunoassay-based analysis of serum samples in a clinical setting (Figure 2). This extends findings by Bradwell et al. who investigated a potential interference of HLC assays with bilirubin, haemoglobin, or triglycerides [7]. We further analysed sera containing different isotypes of MPs with various HLC assays. Our results suggest a cross-reactivity of MP type IgM in the HLC IgG assay (Figure 2) as already described by Bradwell et al. [7]. However, as we were not able to show a normalization of HLC IgG κ/λ ratios after dilution of the respective serum samples, an underlying imbalance in the synthesis of heavy chains type IgG could therefore not be excluded.

Analytical sensitivity of HLC assays was investigated in comparison to IFE as the standard indicator for the presence of MPs [4,11]. Thereby, comparable (HLC IgA and IgM assay) to slightly lower sensitivities (HLC IgG assay) of HLC assays were demonstrated (Figure 3).

A central aim of the present study was to investigate the potential of HLC assays in the detection of MPs in comparison to IFE as the reference test with the idea that equivalence of HLC IgA, IgG, and IgM assays with IFE may allow reduced application of this laborious and observer-dependent standard test. However, limited diagnostic sensitivity of the HLC IgG assay underscores the

role of IFE as the standard indicator for the presence of MPs. In first line diagnostics, HLC analysis may actually be requested especially in cases of unclear patterns in IFE, for example in a case of pronounced polyclonal background.

Two recent publications suggest a potential diagnostic role for HLC analysis as a risk factor for progression of MGUS and as a prognostic marker in myeloma patients [12,13]. In a proof of principle approach, we further investigated a potential diagnostic role of HLC assays in disease monitoring focussing on ScTx IIMM patients. The two patients who achieved an IFE-negative remission had normal HLC ratios (Figure 5C-D); those who failed to achieve IFE-negative remission revealed HLC ratios never reaching reference range (Figure 5A-B). On the basis of normalization of SPE and total IgA concentration in P1 and P2 without evidence of complete remission and the fact that interpretation of total IgA concentration is hindered by overlap between monoclonal and polyclonal background [14], one could speculate that especially the HLC IgA assay may be applied for disease monitoring. We were not able to include transplant patients with IIMM type IgM. Therefore, a diagnostic potential of HLC IgM assays in disease monitoring as illustrated in a recent publication [15] could not be investigated.

A considerable limitation of the present study is our focus on patient sera characterized by a monoclonal band in IFE. Based on this approach, we were not able to investigate whether HLC assays may detect monoclonality in a portion of patient samples characterized by the absence of MPs in IFE. Obviously, based on the low number of ScTx patients included, our conclusion based on the data suggesting a potential role of the HLC IgA assay in disease monitoring is very preliminary and needs further validation.

In conclusion, our comprehensive study including validation of analytical and diagnostic performance of all available HLC assays performed in a clinical setting demonstrated quality criteria suitable for routine application. However, due to limited diagnostic performance especially of the HLC IgG assay, combined application of HLC assays does not represent an alternative to IFE in first line diagnostics. Our preliminary data suggest a diagnostic utility in disease monitoring especially for the HLC IgA assay, which must be confirmed in subsequent large-scale clinical studies.

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Specific contributions of authors to the work:

- Jacqueline Eckold, Wolfram Poenisch, Tim Drogies, and Mathias Bruegel were directly involved in the practical implementation of the present project
- Jacqueline Eckold, Wolfram Poenisch, Juergen Kratzsch, Joachim Thiery, and Mathias Bruegel were involved in designing the study
- Jacqueline Eckold, Juergen Kratzsch, and Mathias Bruegel analysed the data
- Jacqueline Eckold, Juergen Kratzsch, Daniel Teupser, and Mathias Bruegel wrote the manuscript

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Declaration of Interest:

There are no conflicts of interest for any of the authors.

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Correspondence:
Mathias Bruegel, MD
Institute of Laboratory Medicine
Ludwig-Maximilians-University Munich
Marchioninistrasse 15
81377 Munich, Germany
Phone: + 49 89 4400 73220/73209
Fax: + 49 89 4400 78888
Email: mathias.bruegel@med.uni-muenchen.de

Supplement - Table 1. Intra- and interassay precision of Hevylite™ chain assays.

A

	Within-day Reproducibility				Between-day Reproducibility			
	Mean +/- SD (g/L)	CV (%)	Mean +/- SD (g/L)	CV (%)	Mean +/- SD (g/L)	CV (%)	Mean +/- SD (g/L)	CV (%)
Range (g/L)	HLC IgA κ		HLC IgA λ		HLC IgA κ		HLC IgA λ	
11.2 - 44.8	12.6 +/- 0.74	2.97	28.9 +/- 2.38	4.13	12.9 +/- 0.82	3.17	30.8 +/- 3.32	5.39
2.24 - 11.2	9.60 +/- 0.76	3.98	10.4 +/- 0.92	4.42	9.68 +/- 0.86	4.47	10.5 +/- 0.50	2.73
0.56 - 2.24	1.89 +/- 0.06	1.47	1.08 +/- 0.08	3.42	1.96 +/- 0.18	4.33	1.10 +/- 0.12	5.53
< 0.56	0.16 +/- 0.02	4.37	0.12 +/- 0.02	11.1	0.21 +/- 0.08	19.0	0.10 +/- 0.02	11.8

B

Range (g/L)	HLC IgA κ		HLC IgA λ		HLC IgA κ		HLC IgA λ	
11.2 - 44.8	25.0 +/- 1.14	2.28	26.0 +/- 0.40	0.77	25.5 +/- 1.98	3.89	26.0 +/- 0.78	1.50
2.24 - 11.2	9.29 +/- 0.18	1.00	5.89 +/- 0.18	1.55	9.40 +/- 0.32	1.73	6.06 +/- 0.66	5.48
0.56 - 2.24	2.10 +/- 0.16	4.03	2.12 +/- 0.50	11.6	2.10 +/- 0.10	2.24	2.09 +/- 0.34	7.94
< 0.56	0.26 +/- 0.02	3.88	0.48 +/- 0.10	10.0	0.26 +/- 0.01	2.52	0.46 +/- 0.14	15.7

C

Range (g/L)	HLC IgA κ		HLC IgA λ		HLC IgA κ		HLC IgA λ	
11.2 - 44.8	21.2 +/- 0.56	1.32			21.0 +/- 1.28	3.04		
2.24 - 11.2	5.09 +/- 0.14	1.43	4.79 +/- 0.32	3.39	5.22 +/- 0.12	1.06	5.08 +/- 0.68	6.75
0.56 - 2.24	1.39 +/- 0.04	1.40	0.82 +/- 0.02	1.04	1.43 +/- 0.10	3.51	0.82 +/- 0.02	1.63
< 0.56	0.12 +/- 0.01	3.83	0.12 +/- 0.00	1.91	0.12 +/- 0.02	8.55	0.12 +/- 0.00	1.66