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Utility of immunofixation in complementing and empowering serum protein electrophoresis in the diagnosis of paraproteinemia: Experience at a tertiary care center

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Abstract

Background: M' proteins or paraproteins refer to immunoglobulins that are produced by clonal plasma cells and are a characteristic feature of monoclonal gammopathies. Routine electrophoresis on agarose gel and immunofixation can be used to detect immunoglobulin paraprotein (M-protein). We aimed to evaluate the performance of agarose gel electrophoresis alone and in combination with immunofixation for detecting serum M-proteins.

Methods: One hundred and twenty-three patients suspected of paraproteinemia were evaluated. Routine serum protein electrophoresis (SPE) and immunofixation electrophoresis (IFE) protocols were performed. Data from SPE, and SPE-IFE (gel images and electrophoretograms) were collected and reviewed.

Results: 21% cases were confirmed using the SPE-IFE combination, and among them, 33% had positive light chain (λ) only on IFE. Similarly, nine cases with biclonal gammopathy on SPE were characterized by IFE.

Conclusion: IFE can be a confirmatory test in cases where SPE results are not reliable and it can be a complementary test when characterization of the M protein detected on SPE is required.

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Introduction

Paraproteins or M-proteins are the aberrant proteins secreted by the proliferation of clonal plasma cells or lymphocytes. They are observed in conditions such as multiple myeloma, monoclonal gammopathy of undetermined significance (MGUS), Waldenström's macroglobulinemia, solitary plasmacytoma, smoldering multiple myeloma, plasma cell leukemia, heavy chain disease, and amyloidosis. They can be whole immunoglobulins (IgG, IgA, or IgM) or immunoglobulin fragments (usually light chains, very rarely heavy chains) and are fully functional (1). The detection of a paraprotein in blood or urine is a sign of a disorder called monoclonal gammopathy (2). The significance of M-protein is that it can serve as a serologic 'tumor' marker' for the identification of the clone of plasma cells. The first M protein test was described by Dr. William Macintyre and published by Henry Bence Jones (1). Research indicates that paraproteinemias, are relatively widespread and become more frequent as individuals age. Laboratory tests analyzing biochemistry are critical in the evaluation and diagnosis of these conditions in patients (3,4,5).

Serum protein electrophoresis (SPE) on agarose is a relatively less expensive technique used most commonly to screen for changes in serum protein profiles and detect M-proteins. It is dependent on finding a peak that is taller and narrower than albumin. The electrophoretic fraction of gamma globulins in normal serum is a broad, Gaussian distribution (6). This is due to the hundreds of clones of plasma cells that produce immunoglobulins and release them into serum. Sometimes, unusual bands in serum other than M-components can be seen such as transferrin, elevated b-lipoprotein, fibrinogen, and C-reactive protein. Therefore, any suspected band should be considered as a paraprotein or 'M' protein until proven otherwise. The utility of SPE is limited in patients with monoclonal proteins such as IgAs that migrate in the β fraction or hide under the β peak of the protein electrophoresis (7,8). High-resolution SPE (HRE) can be performed using either agarose gel electrophoresis or capillary zone electrophoresis-based techniques (9). HRE permits better detection of low-concentration M-components migrating in the alpha 2, beta, or gamma regions of the gel (10,11). However, the detection limit of SPE/HRE is not sufficiently sensitive, especially in patients with light chain multiple myeloma where the free light chains are quickly removed from the circulation by the kidneys and their concentrations in the serum are very low, or the rarer IgD myeloma cases, where the M-protein appears very small or cannot be detected at all (12). SPE or HRE can only detect the presence of an M protein but it cannot differentiate between the isotypes. The M-protein appears as a confined area of migration in the electrophoresis pattern in monoclonal gammopathies. Although discrete bands in SPE/HRE are rarely false positives, all patients with localized bands and nonhomogeneous distribution patterns should undergo further confirmatory testing.

Immunofixation (IFE) is a gel electrophoresis immunotyping method. The principle of IFE is to anchor, in situ, proteins of a single immunologic species by exposing protein-containing gel to specific antibodies after electrophoresis (13). M-proteins are typically viewed as a restricted immunoglobulin band on IFE and are composed of both heavy and light chains. In current practice, IFE is performed with antisera to IgG, IgA, IgM, total κ and total λ. If a monoclonal light chain is visualized without a corresponding heavy chain, an additional immunofixation gel is performed with antisera to delta and epsilon heavy chains. IFE has an improved sensitivity of approximately ten-fold as compared to SPE (14). Hence, when multiple myeloma or related disorders are suspected, even if the serum protein electrophoretic pattern is negative, it should be followed by IFE. IFE can also assist in distinguishing between a monoclonal and polyclonal rise in immunoglobulins. Studies suggest that monoclonal immunoglobulin can be found by SPE in about 82 percent of myeloma cases (3). When paired with serum IFE, sensitivity rises to 93% as observed in some of the studies (15).

In the present study, we discuss the role of IFE in complementing and empowering SPE/HRE in detecting serum paraproteins from our experience with tests and the resources available at our lab with examples.

Methods

A total of 123 blood samples were collected from clinically suspected cases of paraproteinemia for the study. Serum total protein concentrations were determined using the biuret method (Cobas Integra 400 plus; Roche Diagnostics). Agarose gel electrophoresis was performed using an SAS-MX high-resolution kit by Helena Biosciences. The kit separates serum proteins into five main classes (albumin, alpha 1-globulin, alpha 2-globulin, beta-1 globulin, beta-2 globulin, and gamma globulin) according to charge in an agarose gel. The proteins were stained to allow visualization and quantitative interpretation. Serum samples from electrophoretically normal and clinically healthy subjects were used as quality control material and for assigning fraction demarcations. Immunofixation was performed using the SAS-MX Immunofix kit from Helena Biosciences for identifying the type of heavy (IgG, IgM, or IgA) and light chain (either kappa (κ) or lambda(λ)). The proteins were incubated with specific antisera, and washed and stained to allow visualization of the immunoprecipitate for qualitative interpretation. The gel had separate lanes for testing the immunoreactivity of the sample with heavy and light chain antisera. Results were analyzed using Helena software PT version 3.0. The gels were evaluated independently by two reviewers. The observations by the reviewers were given as definitive evidence of an M protein, definitively ruled out an M-protein, or as an ambiguous pattern. Also, if gel showed an obvious pattern such as acute phase reaction, nephrotic pattern, etc., (15) and monoclonal protein was ruled out by both the reviewers, it was concluded that further studies on the serum for monoclonal protein were not indicated. If a polyclonal increase in the gamma region was observed for a given patient sample, then it was interpreted as a polyclonal increase in the related immunoglobulin. If a suspicious band either in beta, gamma, or alpha regions was observed for a given patient sample, by either or both the reviewers, further

testing with IFE was done to identify the isotype protein. Antisera were used to identify the subtypes of heavy chains (HC) and light chains (LC) based on the presence of discrete bands observed in their respective lanes on the gel. Cases in which a single HC and LC were seen, i.e., one complete immunoglobulin, were classified as monoclonal. When more than one band was observed either in HC or LC or both subtypes, the patient was classified as biclonal. Samples, wherein an additional LC band was seen in the gel but of different mobility, were also taken into consideration and classified as biclonal.

Results

Serum samples from 123 patients clinically suspected of paraproteinemia or high total proteins were received at the Dept of Biochemistry during the period from October 2020 to October 2021. There were 74 (60%) males and 49 (40 %) females. Among them, 55 cases samples showed suspected abnormal bands or bands on SPE and the remaining 68 cases showed either non-specific findings or polyclonal gammopathy.

The 55 cases with suspect bands were taken up for IFE, out of which, 39 (70.9%) were male and 16 (29.1%) were female. The male-to-female ratio was 2.4:1. Age distribution of the cases is demonstrated in Table 1 with Mean ± SD being 56.65 ± 7.5. 29.1% of the cases (n=55) presented with features of renal failure followed by abnormal blood picture and anemia with 21.8% in the study. Clinical features and presenting complaints of the patients are detailed in Table 2. The mean protein concentration was 7.87+ 5.5 (g/dl) and that of albumin was 3.35 + 0.79 (gm/dl). The paraprotein distribution pattern as observed on electrophoresis is represented in Table 3. Monoclonal immunoglobulins (Ig) were demonstrated by SPE and IFE (Figure 1-7). Among the cases (n=55), 49.7%, 32.7%, 5,5%, and 12.7% for IgG, IgA, IgM, and light chain only components respectively with IgG monoclonal type being the highest in number. 21% suspected to be abnormal by either or both the reviewers were confirmed using IFE, among them, 33% had only positive light chain (λ). Furthermore, 19% were IgA + Kappa, 14% were IgG Kappa and 14% where IgM kappa type respectively as can be seen in Table 4. Also, cases with two or more abnormal bands as observed on SPEP were taken up for characterization using IFE as detailed in Table 5.

Table 1. Age distribution of cases with suspected or confirmed 'M' band or bands (n=55)

Age	Frequency	Percent
31 - 40	1	1.8
41 - 50	11	20.0
51 - 60	26	47.3
61 - 70	15	27.3
71+	2	3.6
Total	55	100.0
Mean ± SD	56.65 ± 7.5	

Table 2. Clinical features and presenting complaints (n=55)

Presenting complaint	Frequency	Percent
Bone pains/H/o fractures	5	9.1
Renal failure	16	29.1
Anemia	12	21.8
Fever	11	20.0
Abnormal blood workup	12	21.8

Table 3. Paraprotein distribution of abnormal band or bands on SPE as confirmed by IFE (n=55)

	Frequency	Percent
Light Chain	7	12.7
IgA	18	32.7
IgG	27	49.1
IgM	3	5.5
Total	55	100.0

Table 4. Distribution of paraprotein on IFE (n= 55)

IFE Diagnosis	Confirmed M band		Suspected	
	No.	%	No.	%
IgA +Kappa	1	2.9	4	19.0
IgA +Kappa+Kappa	4	11.8	0	0
IgA +Lambda	0	0	4	19.0
IgA +Lambda + Lambda	5	14.7	0	0
IgG+Kappa	20	58.8	3	14.3
IgG+Lambda	4	11.8	0	0
IgM+Kappa	0	0	3	14.3
Lambda	0	0	7	33.3
Total	34	100	21	100
Chi square = 40.5, p value <0.001				

Table 5. Distribution of monoclonal and biclonal gammopathies (n=55)

SPE	Biclonal		Monoclonal	
	No.	%	No.	%
Confirmed M band	9	100.0%	25	54.3%
Suspected	0	0.0%	21	45.7%
Total	9	100.0%	46	100.0%
Chi square = 6.64, p value = 0.004				

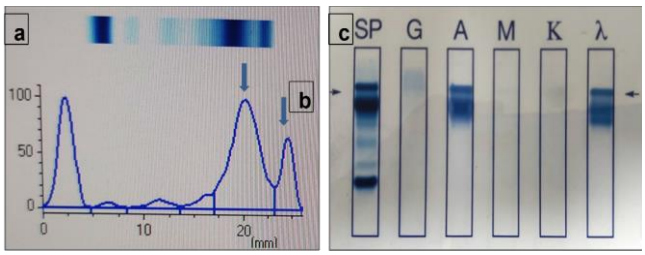

Figure 1. a) SPEP: Two abnormal bands observed in the gamma region b) Densitometer tracing: sharp peaks in gamma globulin region c) IFE: Multiple bands each in immunoglobulin A heavy chain and kappa light chain lanes.

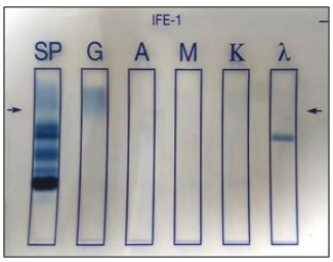

Figure 2. SPEP: One abnormal band in the beta region. IFE shows a light band in the beta with no corresponding heavy chains. This could be a light chain or another intact monoclonal paraprotein. This was ruled out by performing IgD and IgE IFE additionally.

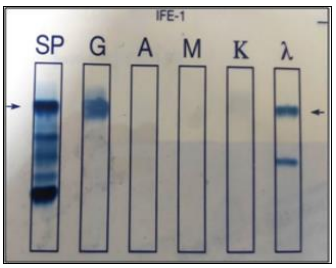

Figure 3. SPEP revealed two abnormal bands, one in the gamma region and another in the alpha 2 - beta region IFE: IgG Lambda monoclonal gammopathy with additional free lambda light chain component.

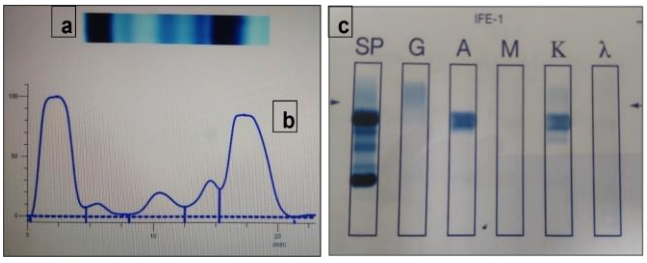

Figure 4. SPEP:(a), (b) Gel picture and densitometry showing an abnormal M-band in the gamma region and a single peak in the gamma region (c) IFE IgA Kappa paraprotein with another band with another band just below it corresponding to the beta region of SPEP but of lesser intensity in the kappa region.

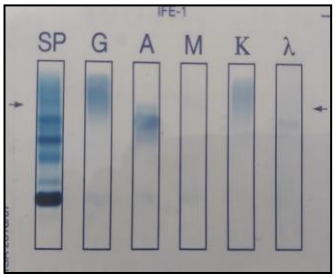

Figure 5. SPEP, One abnormal band in the beta-gamma region. IFE suggestive of IgG kappa and IgA lambda clones. Biclonal gammopathy.

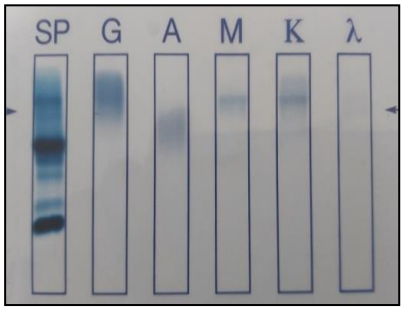

Figure 6. SPEP: Two abnormal bands, one beta and one in the gamma region. IFE is suggestive of IgM kappa monoclonal gammopathy for the band in the gamma region of SPEP.

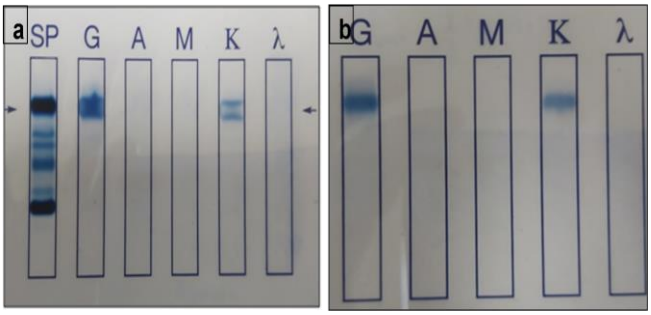

Figure 7. SPEP: conducted in our lab revealed an abnormal band in the gamma region. IFE revealed IgG Kappa type of monoclonal gammopathy. The prozone effect was observed with undiluted and 1:10 diluted samples (comparison of Fig a with Fig b). Urine Bence Jones protein test was positive. Increased kappa light chains and altered kappa lambda ratio were observed in a light chain assay done in an outside lab.

Discussion

The diagnosis of monoclonal gammopathies depends on the identification of monoclonal immunoglobulins or M-proteins in the serum or urine. Techniques for the evaluation of serum M proteins include either gel- or capillary-based electrophoresis along with immunotyping (8). 'M' proteins can be detected as a single discrete band (M band) on an agarose gel and a prominent peak in the densitometer trace by SPEP/HRE. Serum IFE is considered to be the 'gold standard' technique in confirming paraproteinemia diagnosis.

In the present study, out of 123 clinically suspected cases of paraproteinemias, 44.7% (55 cases) showed abnormal or suspect bands on SPE. A study by Chopra et al found that 24.4% of the sample were positive for the M-protein by SPE (16). In the present study, it was observed that 51 to 60 years (47.3%) was the most affected age group with a male predominance of 70.9%. In 45.7% of cases, SPE could not identify the monoclonal nature of the band, and was confirmed by IFE. Distribution of the abnormal bands on SPE as confirmed by IFE was observed to be 49.7%, 32.7%, 5.5%, and 12.7% for IgG, IgA, IgM, and light chain only components respectively with IgG monoclonal type being the highest in number. Further, IFE was also useful in the characterization of the paraprotein in confirmed cases. Among these, 46 were found with a monoclonal and 9 with biclonal components on IFE. Among the cases with monoclonal components observed on SPE, IFE revealed that 49.1% of them were IgG type with 58.8%, 14.3%, and 11.8% of them being IgG kappa, suspected IgG kappa and confirmed IgG lambda types respectively. This was by the study by Tripathy S. et al (7) and Uddin et al (17). IgG is the most common type of paraprotein and usually appears in the gamma region (7). While paraproteins in the gamma globulin region are relatively easy to diagnose by SPEP/HRE, few tend to occur in the beta or alpha globulin region, especially IgM or IgA isotypes. IgA lambda monoclonal gammopathy was the second most common type detected in our lab in 19.0% of cases. A review study of 57 cases by Kyle RA et al. suggests that there is no difference between double/biclonal gammopathy and monoclonal gammopathy clinically and in terms of outcome for the patient (Figure 5) (18). IgA differs from IgG in several characteristics, particularly molecular size heterogeneity. M-proteins of IgA variety may produce peculiar and atypical patterns during electrophoresis which may pose diagnostic difficulties, namely, more anodal migration (close to the β-region) due to lower isoelectric pH, apparent absence of light chains during IFE due to sequestration of light chain epitopes by the quaternary structure of IgA, and confounding by a fibrinogen band due to their unique biological and physicochemical properties (15,19,20). IgA paraproteins also tend to aggregate and form polymers. Polymeric IgA can cause anomalous laboratory findings (e.g., spurious hypercalcemia or overestimation of hemoglobin) that may lead to inappropriate treatment decisions (20). These polymers have a different mobility than the parent monomeric M-protein, thus yielding multiple bands at different positions on SPEP as seen in (Figure 1). Detecting IgA polymers in multiple myeloma is of clinical relevance because such patients are prone to hyperviscosity syndrome. Many centers with limited resources use reducing agents such as beta-mercaptoethanol to resolve the polymeric bands (21). The occurrence of two different M-bands was seen in nine samples on SPEP/HRE in our study and were identified as biclonal gammopathies (Figures 1 and 3). Among them, 11.8% and 14.7% were confirmed IgA +Kappa+ Kappa and IgA + Lambda+ Lambda respectively. Biclonal gammopathy occurs due to clonal expansion of two different tumor cell lines simultaneously. Misra A et al. have reported this finding in 25.1% of such cases in their study (22). However, the identification of two clones increases diagnostic accuracy and credibility. It also helps in understanding the pathogenesis of the disease and in assessing the response of the two clones during treatment and follow-up of patients. The prozoning effect is yet another reason for blotchy or multiple band appearance on SPE or IFE and is often due to antigen excess (Figure 7). This can be eliminated by diluting the sample and analyzing it again (23,24). Light chain-only paraproteins are another entity observed to appear at the beta or even alpha 2 protein fractions due to their smaller size and add to the limitations of SPEP/HRE. Our study detected free lambda light chain components which were either observed on IFE alone (Figure 2) or with another monoclonal gammopathy (Figures 3 and 4). If IFE gel showed a band only in the kappa or lambda lane with no corresponding band in IgG, IgA, or IgM heavy chains, then the sample was tested for IgD and IgE. IgM paraproteinemia is extremely rare. In our study, IgM Kappa monoclonal gammopathy cases were the 13% least with three in number (Figure 6).

Conclusion

Our study suggests that IFE can be a confirmatory test in cases where SPE yields ambiguity and it can be a complementary test when characterization of the paraprotein detected on SPE is required. Further, SPE-IFE can be a good test duo in a limited resource laboratory setup due to their cost effectiveness as compared to newer testing technologies.

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Ethical statement

The study was conducted in the Department of Biochemistry from October 2020 to October 2021 and was approved by the institutional ethics committee (ESICMC/SNR/IEC_F397/01-2022).

Conflicts of interest

The authors declare that there is no conflict of interest as regards this article's publication.

Author contributions

IAS conceptualized the study and provided the study design, statistical analysis, and supervised the work. SGC drafted the paper, contributed to data interpretation, and reported the results. SS performed a critical review. BKR performed the tests. DA contributed to editing the draft. All authors contributed to the discussions and in writing the manuscript.

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