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# Our Laboratory Experience: Comparison of Capillary Electrophoresis/Immunosubtraction and Agarose Gel/Immunofixation

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**Abstract**. Monoclonal gammopathies represent a wide spectrum of related diseases. The common denominator is the presence of a monoclonal protein in the serum or urine, which can be in the form of intact immunoglobulin, immunoglobulin fragments, and/or free light chains (κ and λ). We can detect these abnormalities by immunofixation electrophoresis (IFE) in which specific antibodies are overlaid after electrophoresis and the corresponding immunoglobulin. In our study, we compared gel-based and capillary-based serum protein electrophoresis methods to identify and characterize monoclonal immunoglobulins in serum samples. We analyzed 500 serum samples by Sebia Hydragel agarose gel electrophoresis (AGE)/immunofixation electrophoresis (IFE) and CAPILLARYS 2 capillary zone electrophoresis (CZE)/immunosubtraction (IS). AGE/IFE and CZE/IS had an overall agreement of 98% on serum protein electrophoresis. In our hands, AGE/IFE and CZE/IS had the same specificity for detection of monoclonal proteins; however, CZE/IS seems to be more sensitive than AGE/IFE for the detection of some critical cases. However, CZE/IS is an analytically suitable alternative to AGE/IFE, although laboratories should need to combine, for singular samples, both electrophoretic methods in their clinical routine. A combined use of AGE/IFE and CZE/IS is necessary to lead to an accurate diagnosis and clinically error-free results.

**Keywords**. Serum protein monoclonal component, Immunofixation Electrophoresis, Immunosubtraction Electrophoresis, Laboratory analysis

## 1. Introduction

Several disorders are associated with a monoclonal immunoglobulin, the most common being a monoclonal gammopathy, multiple myeloma, Waldenstrom's macroglobulinemia, and amyloidosis è [1]. The diagnosis of a monoclonal gammopathy is most commonly made after the identification of a serum or urine protein electrophoretic pattern that as being due to a monoclonal immunoglobulin heavy chain and/or light chain [2].

Monoclonal gammopathies represent a wide spectrum of related diseases. The common denominator is the presence of a monoclonal protein in the serum or urine, which can be in the form of intact immunoglobulin, immunoglobulin fragments, and/or free light chains (κ and λ). The abnormality of monoclonal protein may be the alert for undercovered lymphoid malignancy or other pathologic complications [3].

The monoclonal protein serves as a tumor (or clonal) marker in these patients and needs to be monitored to determine whether the clone of plasma cells is expanding [4, 5]. The role in the diagnosis, classification, and management of this disorder is committed by quantifying and immunotyping of serum proteins. In addition, a premalignant lesion, monoclonal gammopathy of undetermined significance (MGUS), is usually diagnosed and followed up by serum protein electrophoresis [6].

Currently, we can detect these abnormalities by immunofixation electrophoresis (IFE) in which specific antibodies are overlaid after electrophoresis and the corresponding immunoglobulin (IgG, IgA, IgM, κ, and λ) is bound and visualized by acid violet or another protein stain. IFE's identification produces a sharp, well-defined band of monoclonal proteins. Broad, diffuse, heavily stained bands with heavy-chain antisera and both κ and λ antisera characterizes polyclonal increase in immunoglobulins. Immunofixation is more sensitive than immunoelectrophoresis [7].

Capillary zone electrophoresis (CZE) is the simplest and most universal capillary electrophoresis (CE) mode applied to separation of proteins. CZE of serum proteins has a huge impact in clinical laboratories [8]. CZE gets the same serum protein separation into six regions. Rather separation is accomplished in a liquid buffer system running through parallel, narrow-bored capillaries consisting of fused silica. The narrow-bored capillaries permit the use of very high voltage [9].

CZE is widely recognized as a powerful analytical technique as AGE but does so without using gels. The sample runs through the narrow capillary tube, and direct protein detection is achieved by a measurement at 200 nm, eliminating the need for staining while improving accuracy and linearity. It is known for its high separation efficiency, short analysis times and low-volume sample requirements, thanks to the complete automatic system [9].

CZE can be combined with immunosubtraction to type the immunoglobulins. The sample is treated with specific antiserum to precipitate and remove the corresponding immunoglobulin [10]. The specific immunotype is shown as a reduction of the peak on CZE.

Nowdays, the use of Capillary Zone Electrophoresis/Immunosubtraction (CZE/IS) in the last few years is more frequent, is a simple method for detecting and immunotyping serum [11]. The capillary immunotyping assay is based on the principle of capillary electrophoresis in free solution. Serum proteins are separated in silica capillaries by their electrophoretic mobility and electroosmotic flow at high voltage in an alkaline buffer [10].

Diagnosis and management of patients with monoclonal gammopathies depend on the accurate identification and characterization of monoclonal proteins [12].

Therefore, to address these critical issues, we analyzed 500 serum specimens by Sebia Hydragel serum protein electrophoresis (agarose gel electrophoresis [AGE]/immunofixation electrophoresis [IFE]) and CAPILLARYS 2 capillary zone electrophoresis (CZE)/immunosubtraction.

The aims of this diagnostic accuracy study were to compare the analysis of monoclonal serum proteins performed by agarose gel electrophoresis and the analysis performed by immunosubtraction. In our study, we also tried to evaluate samples with low peaks of monoclonal components, reporting excellent results.

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## 2. Materials and Methods

### *2.1. Agarose Gel Electrophoresis and Immunofixation (AGE/IFE)*

Protein electrophoresis was performed using the semiautomated Sebia HYDRASYS system with the Hydragel system (Sebia, Lisses, France). The semi-automated HYDRASYS instrument performs migration, immunofixation and staining of the agarose gel for qualitative analysis. The sensitivity of HYDRAGEL IF assay enables the detection and identification of very small monoclonal components. The HYDRAGEL IF assay is based on the principle of agarose gel electrophoresis followed by immunofixation.

After the separation of serum proteins according to their charge, the gel is incubated with different specific antisera targeted against heavy chains (γ, μ, α) and Kappa (κ) and Lambda (λ) free and bound light chains. According to the manufacturer's instructions, the gel is then processed in order to remove the antisera excess prior to the final staining step. Proteins were stained with amido black, and densitometric scanning was performed at 570 nm. The gel is interpreted visually to type all immunoglobulins. Immununofixation was considered the gold standard to identify the monoclonal components.

### *2.2. Electrophoresis/Immunosubtraction (CZE/IS)*

Capillary Zone Electrophoresis/Immunosubtraction (CZE/IS) is relatively easy to perform, does not require special techniques, and can highlight monoclonal proteins such as immunofixation. One of the most used instrument is the Sebia CAPILLARYS capillary electrophoresis systems (Sebia, Lisses, France), which has reliable performance for monoclonal protein identification and quantification [10]. The kit is completely automated and protein profiles are obtained for qualitative analysis.

The assay is based on the principle of capillary electrophoresis in free solution. Serum and/or urine proteins are separated in silica capillaries by their electrophoretic mobility and electroosmotic flow at high voltage in an alkaline buffer. Proteins are directly detected during migration by UV absorbance. Serum and/or urine proteins are mixed with specific antisera directed against heavy chains (γ, μ, α) and light chains (κ and λ). Immunoglobulins specifically react with their corresponding antisera. At the end of the analysis, each antiserum pattern (IgG, IgA, IgM, κ and λ) is automatically overlaid with the ELP (Protein Electrophoresis) curve. Disappearance of the abnormality in the antiserum-treated pattern indicates the presence of a monoclonal protein.

### *2.3. Patient Specimens and Electrophoresis Interpretation*

This retrospective study included 500 fresh patients serum samples were obtained from routine clinical analysis of Lifebrain s.r.l.'s laboratory. The samples at the time of the introduction of the new method in the laboratory were analyzed with both electrophoretic techniques and were acquired during a 90-day period. The study was conducted according to the revised Declaration of Helsinki (1998).

### *2.4. Statistical analysis*

The categorical assignments between AGE/IFE and CZE/IS were analyzed with Kendall's tau-b (τb) correlation coefficient. Using AGE/IFE as the gold standard, the sensitivity, specificity, and positive and negative predictive values of CZE/IS and AGE/IFE were calculated.

## 3. Results

In our study we compared 500 samples of serum immunofixation, analyzed in Lifebrain s.r.l. (Rome, Guidonia; Italy) carrying out a parallel analysis with agarose gel and immunosubtraction.

The statistic data analysis showed us good specificity and high sensitivity of immunosubtraction (or IS), even higher, in some analysis than agarose gel technique (or AGE).
We considered 450 samples with one or more evident monoclonal components, and finally 50 samples that revealed very small monoclonal components, which is not exactly good for using the CZE/IS. The 450 sample with evident monoclonal components, show a good correlation the overall concordance of the 2 methods for band and no-band classification was 98% (Kendall Correlation Coefficient = 0.91, $p< 0.001$) only 7 samples were contrasting. Using AGE/IFE as the gold standard, the sensitivity, specificity, and positive and negative predictive values of CZE/IS and AGE/IFE were calculated and reported in Table 1.
In 4 of the 7 critical cases, we noticed in the agarose gel an IgA presence, which was entirely absent in the immunosubtraction's report.
In this work we want to focus on observing how CZE/IS behaves respect to AGE/IFE in samples with very small monoclonal components even if this technique is not indicated in this case (n=50). Even analyzing these particular cases, we founded a disagreement between the two techniques of 14%; 7 discordant reports out of a total of 50. In particular, in this paper we described some of the 50 cases presenting some agreement or disagreement between the two techniques.

### *3.1. Agreement of AGE/IFE and CZE/IS*

Figure 1 shows some cases where we have agreement with the two methods. In Figure 1A we can observe detection of monoclonal component (MC) of serum proteins by AGE/IFE (left) and CZE/IS (right). The CZE/IS highlights distinctly IgG κ MC and an IgM λ MC; the AGE/IFE was used by validation.
In Figure 1B and 1C, it is possible to see the high performance of CZE/IS even in the detection of anomalies β peaks. In Figure 1B case, the AGE/IFE analysis (left) would have been limited in detection of this anomaly, but trough CZE/IS (right) we could achieve a correct analysis of IgG λ MC in β2 peak. Figure 1C reveals a clear monoclonal component of IgA λ detected by CZE/IS (right), even in the region between β1 and β2 peaks. Specially in this case, the CZE/IS assay was highly performed, despite of AGE/IFE (left), whom analysis would not have been the proper one.
Figure 1D shows good agreement between the two electrophoretic methods, in fact the two IgG λ MCs are evident in both AGE/IFE (left) and CZE/IS (right).

### *3.2. Disagreement of AGE/IFE and CZE/IS*

In the Figures 2A, the AGE/IFE (left) reports confounding results: presence of of IgM κ and IgM λ bands, a not light chain-related IgG and a shadow in IgA. In contrast, the CZE/IS (right) reveals a clear and accurate IgG λ MC (third peak).
Also in Figure 2B, AGE/IFE (left) shows an unclear report; the bands appear confused and not well-defined. Instead, CZE/IS (right) highlights a blunt peak in IgM, which denote presence of an IgM κ MC.
The CZE/IS on the right of Figure 2C shows a marked IgG λ MC, while the AGE/IFE on the left presents inaccurate and confusing bands, which is impossible to associate any exact MC.
In Figure 3, we show how CZE/IS reports excellent results in detect a light IgM λ MC (on the right; pointed by the arrow). Unlike, the AGE/IFE (on the left) is not as effective as CZE/IS, because on gel the IgM band is blurred probably due to human error.
One more session of optimization in order was necessary to increase the technique's resolution, in some AGE/IFE, considered in our work, when the presence of multiple monoclonal components was not well defined.

### *3.3. Problematic detection of IgA*

Some problems have risen in diagnosis of IgA monoclonal component. When the presence of IgA MC is heavier, we have observed a good agreement between the two techniques. Instead when the

presence of IgA MC is not well defined, the CZE/IS can not be considered a valid alternative to AGE/IFE; for these cases, the immunofixation is still the gold standard method.

## 4. Discussion

The CZE/IS technique has a good execution time, its results are obtained in few minutes, just 8 minutes. This is possible because the instrument is fully automated; it requires minimum use of handwork and personnel. In this way, the human and technical errors are minimized, increasing analysis' accuracy. Indeed, the applications of CZE/IS method is technically simpler and more automated than AGE/IFE.

For more than 10 years, IFE has been considered as the gold standard for the analysis of serum proteins MC and for the diagnosis of gammopathies [13, 14], but to date this status has been challenged by CZE/IS [9, 15].

In this study, we showed that CZE/IS was comparable with serum AGE/IFE for identifying serum monoclonal proteins and it may be more sensitive than AGE/IFE. Further, AGE/IFE and CZE/IS data were equivalent in 98.5% of the cases. Anyhow, due to the rapidity of performance and the automatic feature, CZE/IS technique is preferable to AGE/IFE assay.

In this work, we even try to evaluate the samples with lowest monoclonal peaks, achieving excellent results. Also, we could avoid the human errors by the CZE/IS technique, which performs a clear and specific determination of monoclonal components, as it can be seen in Figure 3. A correct and optimized report is an ideal tool that helps the clinician to monitor the efficacy of therapy or it highlights its inadequacy, suggesting other clinical approaches. This indicates that CZE/IS is a good alternative to AGE/IFE.

In the results obtained, 50 samples out of 500 analyzed did not show a clear monoclonal component. Despite the critical conditions, as non-optimal presence of monoclonal component, it was observed that 70% of these 50 samples (15 out of 50) gave a good match with the IFE as reported in Figure 1.

Furthermore, the results with CZE/IS show an excellent resolution even when the monoclonal peak was not well defined. Indeed, in some cases the CZE/IS technique clearly revealed some MCs, despite of AGE/IFE. Especially for MC in β2 peaks, which were not always detectable by analysis on gel, CZE/IS revealed itself reliable.

Our studies have reported a wide range of agreement between gel- and capillary-based methods, with agreement ranging 98.5%. AGE/IS and CZE/IS had similar specificity and sensitivity for detection of monoclonal proteins; however, CZE/IS seems to be less sensitive than AGE/IFE for the detection of small IgA and, possibly, serum light chains. Furthermore, the immunosubtraction method seems technically simpler and more immediate than immunofixation. These features make the CZE/IS a useful alternative approach for AGE/IFE.

## 5. Conclusion

In addition, although AGE/IFE is the gold standard method, for some controversial cases this technique if considered alone is less sensitive than the combination of the two methods.

These features make the CZE/immunosubtraction an alternative approach or in addition to the gold standard method a useful tool for immunotyping. However, it should be specify that the two electrophoresis methods are not mutually exclusive; actually they are both necessary and helpful in the laboratory routine. So, we strongly recommend the combination of both AGE/IFE and CZE/IS, to achieve a good diagnosis and to optimize the daily workflow.

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**Table 1.** Sensitivity, specificity, positive and negative predictive values of CZE/IS vs AGE/IFE.

| | ***Test +*** | ***Test -*** |
|---|---|---|
| ***Disease+*** | 409 | 0 |
| ***Disease -*** | 7 | 41 |

| | | ***Confidence interval at 95%*** | |
|---|---|---|---|
| | | lower limit | upper limit |
| ***Sensitivity*** | 1,000 | 0,990 | 1,000 |
| ***Specificity*** | 0,854 | 0,818 | 0,885 |
| ***Prevalence*** | 0,895 | 0,862 | 0,921 |
| ***Positive Predictive Value*** | 0,983 | 0,965 | 0,992 |
| ***Negative Predictive Value*** | 1,000 | 0,990 | 1,000 |

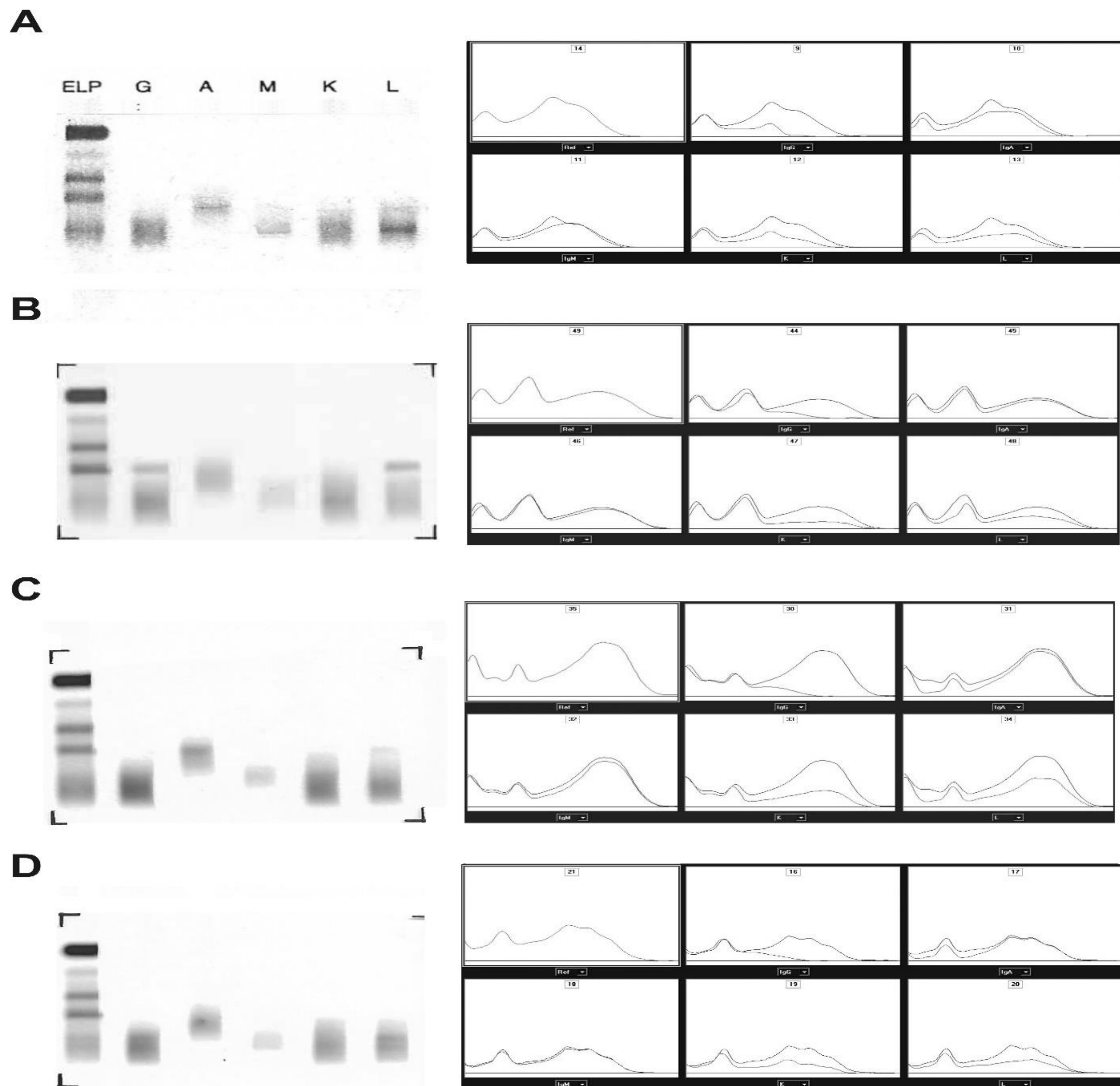
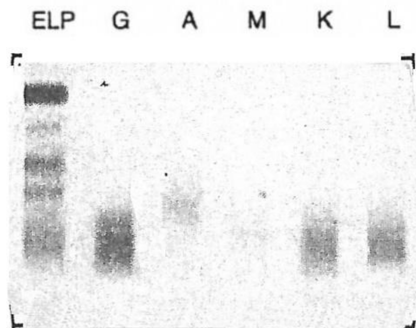
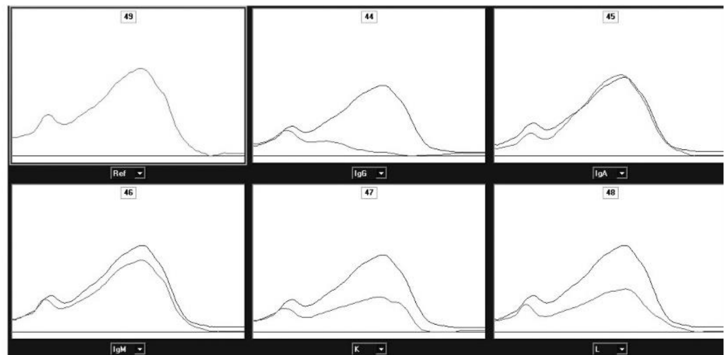



Figure 1. 1A) Representative IgG and IgM case detected by capillary zone electrophoresis (CZE)/immunosubtraction (IS) (right) and by agarose gel electrophoresis (AGE)/Immunofixation electrophoresis (IFE) (left). 1B) Comparison of monoclonal component identification detected in β-2 peak using AGE/IFE (left) and CZE/IS (right). 1C) Comparison of monoclonal component identification detected in region between β1 and β2 peaks using AGE/IFE on agarose gel (left) and CZE/IS (right). 1D) Good agreement between AGE/IFE (left) and CZE/IS (right) in the detection of two IgG λ monoclonal components.

A

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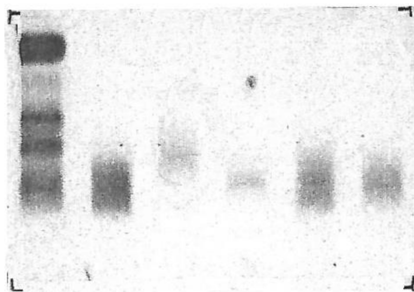

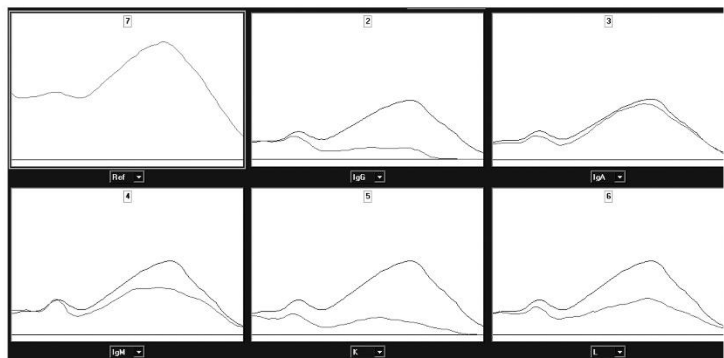


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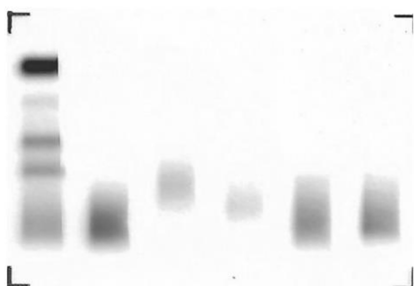

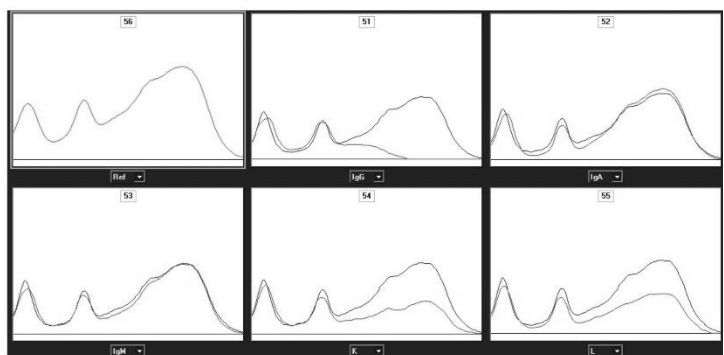


Figure 2. 2A) Representative IgG λ case detected by capillary zone electrophoresis (CZE)/immunosubtraction (IS) (right); on the left confused agarose gel electrophoresis (AGE)/Immunofixation (IFE). 2B) IgM κ case was identified by CZE/IS (right); unclear AGE/IFE analysis (left). 2C) representative IgG λ case showed by CZE/IS (right) and missed by AGE/IFE (left).

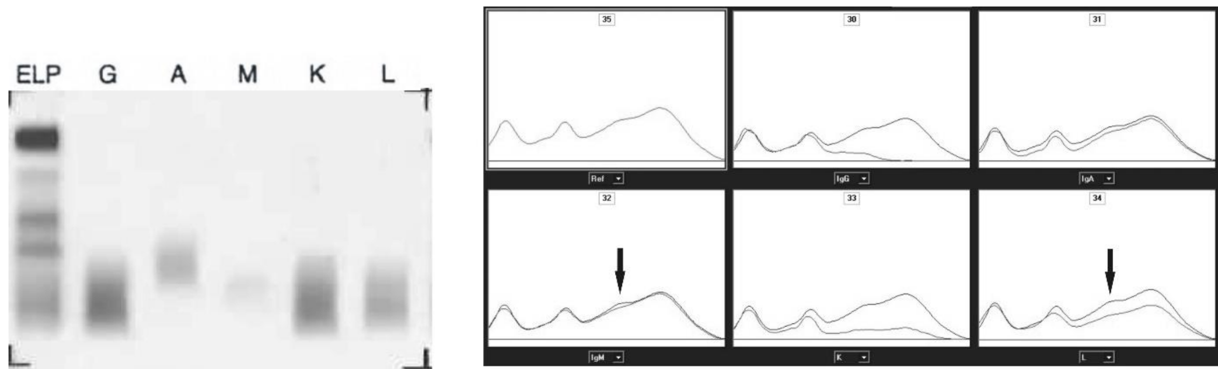


Figure 3. Representative IgM λ case detected by capillary zone electrophoresis (CZE)/immunosubtraction (IS) (right); representative unclear AGE/IFE case (left) because of human error.