CCTC Clinical Chemistry Trainee Council | AACC Better health through laboratory medicine.

PEARLS OF LABORATORY MEDICINE

www.traineecouncil.org

TITLE: Interferences in Protein Electrophoresis

PRESENTER: Anu Singh Maharjan

Slide 1:

Hello, my name is Anu Maharjan. I am a Clinical Chemistry/Immunology fellow at University of Utah, Salt Lake City. Welcome to this Pearl of Laboratory Medicine on "Interferences in Protein Electrophoresis"

Slide 2:

During this talk, I will go over various protein electrophoresis formats, potential sources of interference and approaches for avoiding or resolving such interference. By the end of the pearl, one should be able to identify different electrophoresis techniques employed by clinical laboratories, differentiate various analytical interferences in protein electrophoresis and summarize approaches to minimize the impact of these sources of interference on protein electrophoresis results.

Slide 3:

Gel Electrophoresis is one of the most common electrophoresis techniques performed by applying a sample to a gel support and separating protein through the combination of buffer and electric field that causes a flow of ions between the nodes. Gel electrophoresis consists of a support media, such as agarose, cellulose acetate, or polyacrylamide gels with various pore sizes. Agarose gel is the common support media used in clinical laboratories. Electrophoresis is often carried out in a buffer at pH 8.6, resulting in most proteins having an overall negative charge. Detection of proteins is accomplished through visualization using stains. Examples of the stains used for visualization include Coomassie brilliant blue and amido black. Once the gel is stained, the visible bands can be quantified by densitometry.

Slide 4:

Capillary electrophoresis is a separation technique where electrophoresis is performed in a capillary tube with the application of high voltage. CE consists of a high voltage

power supply, a sample introduction system, a capillary tube, a detector, and an output device. Capillary tubes are usually composed of fused silica with an internal diameter of only 20-100 um. The fused silica contains silanol groups that become ionized in an alkaline buffer creating an electric double layer producing a flow of buffer towards the cathode. This electroosmotic flow causes most of the proteins to travel in the same direction, regardless of their charge. Proteins move from anode to cathode and are detected usually with 200 nm wavelength UV light, but also through electrochemical, fluorescence, conductance, or mass spectrometry methods. CE is popular in clinical laboratories because of fast throughput and utilization of small sample volume.

Slide 5:

Protein electrophoresis separates protein patterns into albumin or globulins. Globulins consist of α-1, α-2, β, and γ. Albumin is the most abundant protein of human serum. α-1 region contains α1-antitrypsin, α-1 acid glycoprotein, α1-antichymotrypsin, and lipoprotein. α-2 region consists of α2-macroglobulin, haptoglobin, and ceruloplasmin. β region can be divided into β-1 or β-2. β-1 consists of transferrin, β-lipoprotein, and C4 while β-2 consists of C3 and fibrinogen. γ region contains immunoglobulins and C-reactive protein. Immunoglobulin A (IgA) can migrate in the β region as well.

Slide 6:

Immunofixation is commonly employed when there is an abnormal protein, known as an M-spike, detected using gel electrophoresis or capillary electrophoresis. Immunofixation electrophoresis characterizes the abnormal bands by applying specific antisera to each lane of the electrophoretic gel. The immune complex is then viewed by staining. Here are examples of immunofixation performed on 3 different samples. The first image shows a normal IFE while the second image shows an IgG κ monoclonal antibody and the third image shows an IgM κ monoclonal antibody. IFE is used to diagnose and monitor different plasma cell dyscrasias such as multiple myeloma and Waldenstrom's macroglobulinemia. IFE is more analytically sensitive, but M-proteins are not quantified using IFE. IFE is also employed in cases where serum protein electrophoresis is unable to quantify small abnormalities, as it can happen with beta-migrating M-proteins or small free light chains.

Slide 7:

One of the most commonly encountered interferences in clinical laboratories is hemolysis, and electrophoresis can be affected by this interference. Hemolysis releases hemoglobin and other cytoplasmic contents of the red blood cell. *In vivo* causes of hemolysis include microbiological agents, pre-eclampsia, hemolytic anemia, and sickle cell disease. *In vitro* causes include use of small bore needles that rupture the red blood cells, excessive vacuum or suction during sample collection, or prolonged sample storage. Hemolysis results in hemoglobin and hemoglobin-haptoglobin complexes to appear as discrete bands in the α2 and β regions. These bands may be misinterpreted as monoclonal proteins when interpreting protein electrophoresis results. The gel

shown in the figure on the right shows the hemoglobin that appear as increased protein in the β region. Visual inspection of the sample can confirm the presence of hemolysis. For hemolysis that is not visible to the eye, IFE can be utilized to rule out the presence of a monoclonal protein. Proper training on blood collection techniques would help minimize such interference. In addition, awareness of the phenomenon and indicating potential interference due to hemolysis in the report could be beneficial to the clinicians.

Slide 8:

Fibrinogen is a normal plasma glycoprotein, which, when cleaved by thrombin to fibrin, forms a fibrin clot for wound healing. Samples that have not clotted properly, especially patients on anti-clotting therapies may present fibrinogen. Fibrinogen will be an interference in protein electrophoresis when the wrong sample type is submitted for analysis. Fibrinogen will migrate to the β/γ region and may be misinterpreted as a monoclonal immunoglobulin. One way to rule out fibrinogen interference is to perform immunofixation. In the figure, electrophoresis shows a peak at the β region, which when reflexed to IFE shows no monoclonal band. Since IFE utilizes specific antisera against the immunoglobulins, IFE will not confirm the presence of a monoclonal protein if the abnormal band was due to fibrinogen. Solutions for fibrinogen interference also include treating the specimen with thrombin or precipitation with ethanol to remove fibrinogen; however, clinical laboratories do not commonly employ these treatments. You can also use test strips, such as Quantofix EDTA, to identify EDTA specimens when there is a suspicion on the sample provided. Providing proper training regarding specimen requirements would help minimize such interference.

Slide 9:

Another exogenous interference encountered in protein electrophoresis, specifically affecting CE, is samples containing contrast dyes. As mentioned, the detection of proteins in CE is often based on UV detection at 200 nm. The radio-opaque agents used in imaging absorb at the same wavelength and will appear as a monoclonal band. Many of these contrast agents interfere with the α2-globulin fraction or less frequently with β2-globulin fraction. In these figures showing CE results, there are 3 different contrast dyes, urografin, telebrix and omnipaque, that show an elevated α-2 region. In a different study, radio-opaque agents at 11 uL to 1 mL of serum, which is the expected concentration after bolus injection for radiographic examination, i.e. 7.5 g/L, led to the appearance of abnormal peak in the CE. Blood collection should not precede for 2-6 days after patients receive contrast media. Since the contrast media interference is due to the UV detection, interference does not tend to occur with other techniques such as gel electrophoresis or immunofixation.

Slide 10:

Antibiotics also cause interference in CE, due to absorbance at UV wavelength, and appear as a monoclonal band in the α or β regions. Here is an example of the antibiotic, piperacillin-tazobactam, administration which generated a small peak at the

anodal site of β-globulin. The figure on the left does not have this peak since the sample was collected before the administration of piperacillin-tazobactam. Other antibiotics that cause interference in CE include ceftriaxone, 5-flurocystosine, and sulfamethoxazole. Immunofixation can be utilized to rule out interference due to antibiotics. Interference may be reduced by drawing the electrophoresis sample when the antibiotic is at trough concentrations.

Slide 11:
For the interferences I have described so far, IFE can distinguish a true monoclonal protein from the interference. However, it becomes more difficult when the interference impacts IFE, which is seen more often with monoclonal antibody therapeutics. Monoclonal antibody therapy such as daratumumab and elotuzumumab (FDA cleared drugs) are used for the treatment of relapsed or refractory multiple-myeloma. Isatuximab is a new monoclonal antibody that is under review for multiple myeloma treatment. All of these monoclonal antibodies are IgG κ antibodies; therefore, infusion of high concentrations will mimic IgG κ M-protein.

Slide 12:
The IFE figures here show the appearance of a monoclonal IgG κ IFE in healthy donor serum sample that has been spiked with different monoclonal antibodies. The interference with protein electrophoresis and IFE may persist for weeks after treatment. Misinterpreted results run the risk of unnecessary additional investigation or disease misclassification.

Slide 13:
There are limited options to confirm and mitigate interference caused by monoclonal antibodies. Because of the number of multiple myeloma patients being treated with daratumumab, there is a daratumumab-specific immunofixation assay, known as DIRA, to confirm and overcome this interference.

The DIRA assay uses a daratumumab-specific antibody to form a complex that causes a shift in the migration of daratumumab on the IFE gel.

The figure shown, demonstrates the assay. Two total serum protein fixation lanes for a baseline and post-treatment of patient with dara are shown in lanes 1 and 2. The assay also uses controls for migration of daratumumab and the daratumumab:anti-daratumumab complex , denoted in lanes 3 and 4. The blue arrow shows the location of the daratumumab control and the orange arrow shows the shift that occurs when daratumumab forms a complex with daratumumab specific antibody.

The antisera used for the IFE assay are specific only for IgG and κ. Baseline serum samples with or without anti-dartumumab are run next to serum samples collected after post-treatment with daratumumab. Red arrows in lanes 7 & 11 indicate a suspected daratumumab interference found in post-treatment. Lanes 8 and 12 are positive for

daratumumab:anti-daratumumab complex, but negative for the suspected band, indicating that the sample is DIRA negative, which means absence of monoclonal protein. If there were residual IgG κ endogenous M protein, a second band would remain in that suspected region. This will correspond to a positive DIRA test, indicating a presence of monoclonal protein. DIRA is limited in that the assay is only designed to distinguish daratumumab and not other monoclonal antibody therapeutics.

Slide 14:
An alternative to DIRA test is MALDI-TOF mass spectrometry, also known as MASS-FIX. Based on the mass of daratumumab, the technique can distinguish daratumumab from the patient's endogenous M-protein. However, this technique is still prone to false negative results for approximately 16% of patient samples as shown in a study by Moore et al in 2019. MASS-FIX, therefore, cannot distinguish all samples because of a poor resolution of a linear MALDI-TOF instrument.

There is another developmental mass spectrometry technique called miRAMM – Monoclonal-immunoglobulin-rapid-accurate mass measurement. miRAMM differentiates an endogenous M-protein from monoclonal antibodies. The assay is based on the identification of mass spectra of the light chain portions of immunoglobulins that are converted into molecular masses of each light chain variant. miRAMM can distinguish differences in molecular masses of M-proteins within 1 Da. This technique distinguishes broad spectrum of monoclonal antibody therapeutics. However, it's not used in the clinical laboratory yet.

Slide 15:
Here is an example of miRAMM, distinguishing M-protein from daratumumab in a patient sample spiked with different concentrations of daratumumab. A neat serum sample with a distinguished IgG-κ M protein is subjected to different concentration of daratumumab, and analyzed with IFE and miRAMM. The serum sample is diluted with different concentration of daratumumab to get an overall M-protein of 0.3 g/dL. The top panel of IFE results show that there is no difference in the image output at different concentrations of daratumumab. The protein electrophoresis shows that overall 0.3 g/dL M-protein is maintained even though the daratumumab:M-protein ratio changed. Finally, the samples with different concentration of daratumumab:M-protein is analyzed with miRAMM. miRAMM clearly distinguishes the peak for M-protein and daratumumab. It shows that the daratumumab peak elevates with increasing concentration of daratumumab while M-protein peak decreases.

Slide 16:
In addition to DIRA and mass spectrometry assay to overcome therapeutic antibody interference, a new technology called Antigen Specific therapeutic monoclonal Antibody Depletion Assay (ASADA) seems promising in depleting interference by therapeutic antibody. ASADA consists of magnetic beads coated with antigens against the specific

therapeutic antibodies. To deplete daratumumab, the magnetic beads are coated with His-tagged CD38. Similarly, to deplete elotuzumab, the magnetic beads are coated with His-tagged SLAMF7. The use of the magnetic beads show potential for multiplexing for different therapeutic antibodies. In a study by Liu et al, ASADA was highly specific in depleting daratumumab in 12 samples known to have daratumumab therapy. Only 1 patient sample confirmed with daratumumab therapy did not show daratumumab depletion after ASADA treatment. That sample had high concentration of endogenous IgG/k that co-migrated with daratumumab, causing persistent cathodal IgG/k even after ASADA treatment.

Slide 17:
This table summarizes the interferences that were covered along with affected methods and ways to resolve the interference. Interfering agents such as hemolysis, fibrinogen, contrast dyes, and antibiotics affect gel electrophoresis and capillary electrophoresis. These interference issues can be resolved using immunofixation. Monoclonal antibody therapy is one of the newest interfering agents that affects not only gel electrophoresis and capillary electrophoresis, but also immunofixation. Several methods such as specific-mAb shift assay, mass spectrometry, or antigen specific therapeutic monoclonal antibody depletion assay (ASADA) can be utilized to overcome interference by monoclonal antibody therapy.

Slide 18:
Recognizing different interferences will help in determining the right approach to troubleshooting the issue. Many of the interferences can be resolved by using IFE, but monoclonal antibody therapeutics can interfere with accurate interpretation of IFE. Therefore, use of DIRA, mass spectrometry assays, or ASADA, if available, may resolve interference due to monoclonal antibody therapeutics.

Slide 19: Here are the references that I used to prepare for this talk.

Slide 20:

I do not have any disclosures for this presentation.

Slide 21: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on “Interferences in Protein Electrophoresis.”