Leakage of cerebrospinal fluid (CSF) as rhinorrhea or otorrhea is a serious condition that can result from trauma, invasive tumors, or surgical procedures. Without appropriate treatment, CSF leaks can lead to life-threatening cases of meningitis, intracranial hypotension, or pneumocephalus. Identification of CSF in non-native sites requires immediate surgical intervention, usually in the form of endoscopic endonasal or transcranial surgery.

A variety of methods exist to detect CSF leakage: high-resolution computed tomography, magnetic resonance imaging, intrathecal or topical fluorescein, and biochemical tests such as β2-transferrin. The major isoform with 4 sialic-acid groups is β1-transferrin, the predominant form of transferrin in serum. In contrast, asialotransferrin, or β2-transferrin, has no sialic-acid side groups and is confined to the CSF, aqueous humor, and perilymph. Therefore, β2-transferrin in nasal or aural fluid serves as a marker for CSF leakage. β2-transferrin can be resolved from the other transferrin isoforms because differences in sialic acid content change the isoelectric point of the protein, allowing separation by agarose electrophoresis.

Current methods for detecting β2-transferrin are often inadequate to isolate a single β2-transferrin band from other isoforms en masse. We have observed that the separation between these bands was sometimes poor, leading to indeterminate or false-negative results. We therefore sought to increase the resolution of the assay. In
this study, we evaluate a morpholinopropanesulfonic acid (MOPS)–histidine solution as a novel buffer for this purpose. The MOPS-histidine solution exhibits a pH close to the isoelectric point of transferrin, increasing the differential charge between the isoforms and theoretically enhancing their electrophoretic separation.¹³

Materials and Methods

Patient Specimens

We obtained 20 patient specimens of nasal or aural fluid from routine submissions for β₂-transferrin testing in the Clinical Immunopathology Laboratory at the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. The specimens were collected from 9 women and 11 men who ranged in age from 21 years to 71 years (mean, 51 years; median, 52 years). All specimens had been recently tested using the agarose electrophoresis in a barbital buffer method. We used CSF and serum as the positive and negative controls, respectively. The use of leftover patient specimens for research purposes was approved by the Institutional Review Board of our institution.

Electrophoresis

We carried out electrophoresis using glass slides coated with 2 mm thick 0.75% agarose (SeaKem slides; Lonza Group Ltd., Basel, Switzerland). Also, we used Pierce phosphate-buffered saline (PBS; Thermo Fisher Scientific Inc., Waltham, MA) to make 1:1, 1:5, 1:10, and 1:50 dilutions of patient specimens. Applications of 1.5 μL of CSF positive control specimens, patient specimen dilutions, and purified human serum negative control specimens were applied to the agarose gel with a SPIFE Urine/CSF template (Helena Chemical Company, Collierville, TN). We ran the agarose-coated glass slides on a vertical electrophoretic platform (Thermo Scientific OWL Dual-Gel Vertical Electrophoresis System, Thermo Fisher Scientific Inc.). Gauze strips soaked in 30 mM MOPS solution and 25 mM L-histidine running buffer (pH 6.6) were applied to the ends of the agarose and placed in the positive and negative ion chambers. We ran electrophoresis at 400 volts for 25 minutes in the MOPS-histidine buffer and 275 volts for 40 minutes in the barbital buffer. Except for the electrophoretic time and voltage, all other procedural steps were identical.

Western Blot

After electrophoresis, a 0.45-μm pore nitrocellulose membrane (Osmonics NitroPure, Krackeler Scientific Inc., Albany, NY) moistened with deionized water was placed on the agarose gel. We then placed a combination of dry filter paper and nontextured paper towels on top of the membrane as we applied pressure to the entire sandwich using an Immuno SuperPress (Helena Chemical Company) for 25 minutes. The membrane was subsequently incubated for 25 minutes in 1% Difco skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ) and tris(hydroxymethyl) aminomethane (Tris)–buffered saline blocking solution. A 15-minute incubation with a 1:5000 dilution of goat antihuman transferrin antibody in a 1% bovine serum albumin (BSA), 4% polyethylene glycol (PEG), and 0.1% Tween-20, Tris-buffered saline solution was followed by three 5-minute washes with 4% PEG and 0.1% Tween-20 in Tris-buffered saline. We then incubated the nitrocellulose with a 1:5000 dilution of peroxidase-conjugated swine antigoat antibody in a 1% BSA, 4% PEG, and 0.1% Tween-20 in Tris-buffered saline solution for 15 minutes. After three 5-minute washes with 4% PEG and 0.1% Tween-20 in Tris-buffered saline, the membrane was developed by incubation with a TM Blue precipitating reagent (SeraCare Life Sciences, Inc., Milford, MA). We halted development of the nitrocellulose membrane by several washes with deionized water.

Neuraminidase Cleavage of Sialic Acid Residues

We used neuraminidase from type III Vibrio cholera to determine the sialic acid content between different bands on the developed nitrocellulose membrane. A 0.2 mg/mL transferrin solution was prepared using partially saturated purified human transferrin and PBS. We mixed the transferrin solution with neuraminidase in 1:1, 1:5, 1:10, and 1:50 dilutions. These mixtures were incubated in a 37°C water bath for 10 minutes and then placed in a freezer. The transferrin solution and each of the neuraminidase-treated transferrin specimens were diluted with 1:5 PBS. We analyzed the specimens via agarose electrophoresis in the same manner as patient specimens, as detailed earlier herein.

Iron Saturation

We determined the effect of iron level, as detected by the assay, by preparing iron-saturated and iron-reduced CSF
specimens. Iron saturation was achieved by incubating CSF in excess Fe$^{3+}$. Iron-free CSF specimens were made by mixing 6 μL of CSF with 1 μL of 100 mM Na$_2$-ethylene-diaminetetraacetic acid (EDTA) in a method similar to that described by Altland et al.$^{12}$ We analyzed the specimens via agarose electrophoresis in the same manner as patient specimens, as detailed earlier herein.

**Data Analysis**

Concordance for the identification of the $\beta_2$-transferrin band was assessed qualitatively. A $\beta_2$-transferrin band was categorized either as present or not present. Discrete values of each assay were compared to evaluate the simple concordance.

**Results**

Electrophoretic Analysis of $\beta_2$-Transferrin in CSF

The MOPS-histidine buffer produced 5 distinct CSF transferrin bands (Image 1). The $\beta_2$-transferrin band is the furthest (anodal) migrating isoform and is not present in the negative serum control (Image 1, lane 1, arrowhead). In comparison, electrophoresis with the standard barbital buffer yields only 2 bands, with the leading band representing $\beta_2$-transferrin (Image 2).

To confirm that the additional bands observed with the MOPS-histidine buffer represented other sialic acid transferrin isoforms, we performed neuraminidase treatment similar to the technique described by Parker and Bearn.$^{14}$ The successive cleavage of sialic acid groups with exposure to neuraminidase at different concentrations verifies the presence of sialic acid transferrin isoforms. As each sialic acid is removed, the partially desialylated transferrin is able to migrate further in the agarose gel. This gives the appearance of a stepwise pattern of bands when increasing concentrations of neuraminidase are used. Cleavage of sialic acid residues proceeds until $\beta_2$-transferrin or asialotransferrin remains (Image 3).

To evaluate the effect of iron loading on the migration patterns of the transferrin isoform bands, we compared Fe$^{3+}$-saturated and iron-free CSF specimens. The isoelectric point of transferrin is altered when iron is bound, thus potentially altering the movement of the protein through the agarose gel. However, no appreciable differences were noted (Image 4).

**Comparative Evaluation of Clinical Specimens**

We tested 20 patient aural or nasal fluid specimens with both buffers; 17 results were concordant (8 positive, 9 negative). Representative patient specimens with the MOPS-histidine buffer are shown in Image 5, demonstrating 2 positive specimens (lanes 2 and 4) and 2 negative specimens (lanes 6 and 8). The 3 remaining discrepant specimens all tested negative with the barbital buffer and tested positive with the MOPS-histidine buffer. However, 2 of these 3 specimens later tested positive with the barbital buffer when a new specimen from each of these same patients was resubmitted several days later. An additional specimen was not received for comparison from the final patient with discordant results. A representative specimen illustrating the early detection of $\beta_2$-transferrin with the MOPS-histidine buffer is detailed in Image 6. The use of a MOPS-histidine buffer in lane 2 clearly shows an extra band (arrowhead) representing $\beta_2$-transferrin, compared with the barbital buffer (lanes 7, 9, and 11).

**Discussion**

Transferrin is an iron-transporting glycoprotein with 2 N-linked glycan chains and 2 sites for reversible Fe$^{3+}$ binding.$^{15}$ Smithies$^{16}$ first reported transferrin genetic heterogeneity, differences in iron content, and variability in sialic acid content in transferrin isoforms. More than 30 genetic variants of transferrin have been documented; however, deviations from the normal phenotype are rare.$^{17}$ Differences in iron content are achieved by varying the number and position of bound iron ions.$^{16}$ The most useful method to detect transferrin isoforms is by sialic-acid content.

Each of the 2 N-linked glycan chains of transferrin terminates in a variable number of sialic-acid residues. Nine isoforms are possible (from 0 to 8 total sialic acid groups), each with an isoelectric point differing by approximately 0.1 pH units.$^{6}$ The liver produces sialylated isoforms, which are post-translationally modified by glucuronyltransferase and sialidase to add or remove sialic-acid residues, respectively. The isoform with 4 sialic acid
Resolution of transferrin isoforms with the morpholinopropane-sulfonic acid (MOPS)–histidine buffer. Multiple transferrin isoforms are visualized when cerebrospinal fluid is run on agarose electrophoresis with the MOPS-histidine buffer (lane 1), compared with the negative serum control (lane 2). The arrowhead demonstrates the β₂-transferrin band, which is absent in the negative control lane.

β₁-transferrin, is the predominant form of transferrin in human serum. The sialylated isoforms can pass through the blood-brain barrier where they encounter the sialic-acid–removing enzyme neuraminidase. This ensures that the isoform with no sialic-acid groups, β₂-transferrin, predominates in the CSF. The loss of sialic acid residues prevents the movement of β₂-transferrin across the blood-brain barrier, and therefore, β₂-transferrin is confined to the CSF, aqueous humor, and perilymph. The differences in sialic-acid content can be visualized via a number of methods, making β₂-transferrin a valuable biological marker for CSF.

Separation of more than 2 transferrin isoforms has previously been observed with other laboratory methods, such as denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing. To our knowledge, ours is the first report of this phenomenon in native gel electrophoresis. The typical electrophoretic pattern of CSF yields 2 bands in barbital buffer, which is the standard buffer used in many clinical laboratories, including our institution. The leading band represents β₂-transferrin, with the remaining isoforms migrating as a separate, single band. The MOPS-histidine buffer can resolve multiple bands corresponding to various transferrin isoforms that differ by sialic-acid content. To confirm that the additional bands were present due to variations in sialic-acid content, we treated purified human transferrin with neuraminidase. The enzyme reduced the total number of sialic acid residues, resulting in a stepwise pattern consistent with previous reports of neuraminidase-treated transferrin. The electrophoretic pattern indicates that each band likely corresponds to a single or group of transferrin isoform(s) with a different number of sialic-acid residues. When MOPS-histidine is used as a buffer, there are differential interactions between the buffer and the various transferrin isoforms, due to the similar pHe of the MOPS-histidine buffer and isoelectric point of transferrin. However, complete resolution of all 9 isoforms is not

Image 1
Resolution of transferrin isoforms with the morpholinopropane-sulfonic acid (MOPS)–histidine buffer. Multiple transferrin isoforms are visualized when cerebrospinal fluid is run on agarose electrophoresis with the MOPS-histidine buffer (lane 1), compared with the negative serum control (lane 2). The arrowhead demonstrates the β₂-transferrin band, which is absent in the negative control lane.

Image 2
Resolution of transferrin isoforms with the barbital buffer. The standard barbital buffer only allows visualization of β₁- and β₂-transferrin bands when testing cerebrospinal fluid. The positive control is demonstrated in lane 1 with the β₂-transferrin band (arrowhead). The negative control (lane 2) lacks the β₂-transferrin band.
observed and may reflect clustering of similar isoforms in a single band or a concentration below the threshold of detection.

The effect of iron saturation was also evaluated, because the number and position of bound iron can change the isoelectric point of transferrin and potentially alter its electrophoretic movement. We observed no difference between iron-saturated or desaturated CSF when run with the MOPS-histidine buffer. This observation suggests that grossly bloody specimens containing iron from hemolyzed red blood cells should not affect the assay.

The MOPS-histidine solution has high resistance, yielding currents 10-fold smaller than those produced during electrophoresis with the standard barbital buffer. Application of high voltage (eg, 400 volts) without overheating the agarose gel confers 2 advantages over the typical barbital buffer. First, a large separation between transferrin isoforms can be achieved with short electrophoretic runs. The MOPS-based buffer and the increased voltage setting also may also affect the migration pattern of the nontransferrin proteins. Serum specimens demonstrate a large region of staining that obscures the β₁-transferrin band and is most likely due to nontspecific staining of superimposed nontransferrin proteins. Second, a cooling apparatus or cold environment is not needed, contrary to what is often called for in other procedures, to reduce heating of the gel.

We compared the performance of the novel buffer with the standard method on patient specimens submitted to our laboratory for CSF testing. Seventeen of 20 specimens (85%) showed concordant results via the 2 methods. Two of the remaining 3 patient specimens yielded negative results initially, via the hospital-based assay, but tested positive with the MOPS-histidine buffer. Several days later, when a second specimen was submitted from 2 of the same patients, the barbital-based method detected a β₂-transferrin band. This finding suggests that the MOPS-histidine buffer may increase the sensitivity of testing.
Improved detection of cerebrospinal fluid (CSF) leakage with the morpholinopropanesulfonic acid (MOPS)–histidine buffer. A patient specimen was identified as negative via the use of a barbital buffer in lanes 7, 9, and 11, compared with repeat testing with 1:1, 1:5, 1:10, and 1:50 dilutions in a MOPS-histidine buffer in lanes 2 through 5. Lanes 1, 8, and 10 are positive CSF controls (arrowhead indicates $\beta_2$-transferrin).

**Image 5**
Representative patient specimens with the morpholinopropanesulfonic acid (MOPS)–histidine buffer. Representative patient specimens submitted for cerebrospinal fluid (CSF) testing with positive CSF controls in lanes 1, 3, 5, and 7 with corresponding $\beta_2$-transferrin bands (arrowhead). Lanes 2 and 4 represent positive patient specimens with distinct transferrin bands, including the $\beta_2$-transferrin band. Lanes 6 and 8 represent negative specimens.

**Image 6**
Improved detection of cerebrospinal fluid (CSF) leakage with the morpholinopropanesulfonic acid (MOPS)–histidine buffer. A patient specimen was identified as negative via the use of a barbital buffer in lanes 7, 9, and 11, compared with repeat testing with 1:1, 1:5, 1:10, and 1:50 dilutions in a MOPS-histidine buffer in lanes 2 through 5. Lanes 1, 8, and 10 are positive CSF controls (arrowhead indicates $\beta_2$-transferrin).
An added benefit of the MOPS-histidine buffer became apparent when bloody specimens were tested. In non-bloody specimens, visualization of $\beta_1$- and $\beta_2$-transferrin bands is typically straightforward. However, proteins in blood co-migrate with transferrin and separation of the 2 bands can be difficult to interpret. Antibodies used to detect transferrin are typically overexpressed near the $\beta_1$-transferrin band because $\beta_1$-transferrin is the predominant form produced. These antibodies can bleed into the adjacent area where the $\beta_2$-transferrin band is found, obscuring the identification of transferrin isoforms. This interference commonly results in repeat testing or an indeterminate result, which can delay management or impact the treatment plan for a patient. Our experience has been that patients with head trauma having CSF leaks are contaminated with blood. Resolving transferrin isoforms with the MOPS-histidine buffer would prevent inconclusive results when the $\beta_2$-transferrin band obscures the adjacent $\beta_1$-transferrin band, allowing earlier detection of CSF leaks.

The procedures for both assays are nearly identical and could easily replace an already existing method using the barbital buffer. The MOPS-histidine buffer only differs in the electrophoretic voltage and run time. Transitioning to using the MOPS-histidine buffer would decrease the overall turnaround time by 15 minutes and does not require additional training of laboratory staff. Improvements in reporting positive results more quickly have the potential to improve patient care. Additionally, the reagents currently being used are compatible with the newer method. The potential increase in sensitivity and decreased run time makes this an appealing method. Future studies including more patient specimens will clarify the clinical role of the MOPS-histidine buffer.

Our study was limited by the small patient sample size. The clinical significance of resolving additional transferrin isoforms in the CSF with the MOPS-histidine buffer requires further evaluation. This study was not designed to identify the lower limit of detection, sensitivity, or specificity. Further testing to evaluate these parameters would be useful to determine their clinical utility.

In summary, modifying a clinical agarose-gel electrophoresis assay with a MOPS-histidine buffer increases the resolution of transferrin isoforms. The technique may have an improved level of sensitivity for detection of transferrin isoforms. LM

References


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