Rapid Method for $\beta_2$-Transferrin in Cerebrospinal Fluid Leakage Using an Automated Immunofixation Electrophoresis System

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Background: $\beta_2$-Transferrin ($\beta_2$ trf), is a desialated isoform of transferrin found only in cerebrospinal fluid (CSF), ocular fluids, and perilymph. In aural, nasal, and wound drainages, this protein is an important marker of CSF leakage. Immunofixation electrophoresis (IFE) on agarose gels is a widely accepted qualitative technique for detection of small amounts of $\beta_2$ trf, but disadvantages include lengthy transfer immunoblotting techniques or the requirement of at least 2 mL of sample.

Methods: Using eight applications of unconcentrated sample on high-resolution agarose gels with an automated electrophoresis system (Helena SPIFE 3000), we developed a rapid method for $\beta_2$ trf. Evaluation studies included reproducibility of migration distance (mm), limit of detection, specificity, and concordance of results compared with those reported by a reference laboratory. Neuraminidase-treated serum was the source of $\beta_2$ trf for our sensitivity and specificity studies. Transferrin was measured by rate nephelometry.

Results: The 2.5-h procedure demonstrated reproducible migration (CV <2.5%) on five lots of gels. Detection of $\beta_2$ trf at 0.002 g/L in an unconcentrated sample was attributed to reproducible application, quality of the anti-trf antiserum, and a sensitive acid violet stain. Our $\beta_2$ trf findings (two negative and five positive) in seven available clinical samples agreed with the reference laboratory results. In 12 months after its inception, this test was ordered 48 times vs 13 in the previous year when testing was sent out.

Conclusion: This method provides physicians with a rapid, reliable aid in the diagnosis of suspected CSF leakage, as described in a case report.

Transferrin (trf)$^3$ is an iron-binding glycoprotein found in several polymorphic forms in the serum and other body fluids (1–3). The “tau” isoform of trf is the neuraminidase-induced product that has lost one of its four neuraminic acid moieties and occurs in the cerebrospinal fluid (CSF), perilymph (4), and ocular fluids (5) but rarely in the serum. This is a desialated isoform, or $\beta_2$ trf, that is useful in differentiating CSF from serum, which typically contains the fully sialated $\beta_1$ trf. Individuals who carry a rare allelic variant (6), chronically abuse alcohol, or have severe liver disease (7–10) may have additional desialated isoforms of trf in serum. The heterogeneity of trf variants as potential sources of false positives in testing for CSF leakage has been discussed by others (1, 6, 11, 12).

Physicians frequently encounter patients with rhinorrhea or otorrhea from a variety of infectious, allergic, or inflammatory etiologies. Less common but more serious is CSF rhinorrhea or otorrhea. CSF leakage may occur in several conditions involving accidental trauma, surgical trauma, neoplasm, or congenital disorders, or it may occur spontaneously with fistulas (13). A rapid, definitive diagnosis is critical to avoid the serious risks of meningitis, pneumocephalus, or brain abscess. Most diagnostic techniques available to clinicians are invasive and require lumbar punctures to confirm the clinical suspicion of CSF leakage. Sensitive, specific, and rapid testing can eliminate the need for invasive and costly procedures such as computed tomography cisternogram or intrathecal fluorescein staining. The laboratory evaluation of drainage fluids to detect CSF leakage is performed by separation methods that rely on the compositional differences be-
between CSF and other body fluids. Testing for glucose, total protein, or prealbumin in nasal, aural, or other drainages is noninvasive but nonspecific and is no longer recommended (1, 3). On the other hand, testing for β-2 trf in drainage fluids permits the physician to screen for, manage, or even diagnose a CSF leak.

Although many methods have been reported (4–6, 8, 10–12, 14–17) for detecting trf isoforms for conditions in addition to CSF leaks, the most common qualitative technique in the clinical laboratory is immunofixation electrophoresis (IFE). The result is interpreted as positive if two trf protein bands, representing β-1 and β-2, are seen in the β-globulin zone of the stained pattern. At an alkaline pH, the β-2 trf band migrates slower (toward the cathode) and stains less intensely than the proportionally dominant, faster-moving (anodal) β-1 trf band (1, 4, 11, 12, 15–17). Specificity by IFE is obtained first by sufficient separation of the isoforms electrophoretically and then by precipitation of the trf variants with anti-trf antiserum. To achieve good sensitivity and compensate for the inherently low trf concentration in CSF (1, 3, 7, 11, 12, 15), many laboratory procedures require that 2–5 mL of the suspected fluid must be concentrated as much as 10-fold to have a suitable sample for IFE. On the other hand, multiple applications of the unaltered sample can replace the concentration step, thereby obviating the need for a large volume of the original sample and the time to concentrate it.

Recently when our clinical services laboratory acquired an automated electrophoresis system (SPIFE® 3000; Helena Laboratories) to consolidate routine electrophoresis and immunofixation procedures on one workstation, we considered providing a method on the same platform for β-2 trf in CSF leakage. The analytical conditions of IFE that can improve sensitivity and specificity are those that affect the amount of sample loaded, the amount applied, electrophoresis voltage and time, the antiserum, and the protein detection stain. The “user test” option of the newly acquired system was the key for programming a specific set of automated electrophoresis instructions that enabled the studies described below.

Our work had three principal aims: (a) to meet performance specifications, guided by previous reports, that characterize a specific, highly sensitive, reproducible method; (b) to have a method that produces results comparable to other modern methods in clinical laboratories (1); and, (c) to meet efficiency requirements and staffing limitations in this laboratory by consolidation of electrophoresis procedures with present-day automation. The evaluation of this method included studies to measure the following performance characteristics: reproducibility of migration distance; specificity and detection limit for β-2 trf; verification of results by use of concentrated and unconcentrated samples; and accuracy by comparison of our results for β-1 trf and β-2 trf results with the results obtained for the same patient samples reported by a reference laboratory.

Case Report

A 40-year-old white male presented to our institution in January 2004 for evaluation of possible CSF rhinorrhea. He had previously been diagnosed with a pituitary macroadenoma and underwent transsphenoidal surgery with partial tumor resection elsewhere in June 2000. He subsequently developed a CSF leak that resolved after treatment with a lumbar drain. He then underwent radiation from September to October 2000 for residual adenoma. His CSF leak then recurred. He underwent a revision transsphenoidal CSF leak repair with subsequent leak 1 month later. He underwent a third surgery in November 2002 to repair his CSF leak. From June through December of 2003, he underwent 90 dives of hyperbaric oxygen in an attempt to get the radiated field to heal and also to seal the CSF leak. After hyperbaric oxygen, his clear rhinorrhea had improved, and it was no longer certain that he had a CSF leak. In our Otolaryngology Clinic, endoscopic exam failed to detect a definitive skull base defect or CSF leak. Radiographic studies demonstrated residual pituitary adenoma in a radiated bed of scar tissue with no obvious bony skull base defect. He was able to collect ~0.5 mL of fluid from his nose. Subsequent laboratory analysis, as described in this report, of the fluid was positive for β-2 trf and β-1 trf (similar to the depictions in Fig. 1).

Given the positive laboratory result for β-2 trf and the previously negative radiographic studies, the patient was diagnosed with a low-flow, intermittent CSF leak. It was felt that further radiographic studies would likely be negative as well; thus the patient underwent endoscopic examination with intrathecal fluorescein. Intraoperative localization of his CSF leak and skull base defect by intrathecal fluorescein confirmed the clinical diagnosis of CSF rhinorrhea and localized the defect to the posterior olfactory cleft, adjacent to the original extent of the tumor. Endoscopic repair of the defect was performed successfully with clinical resolution of his rhinorrhea. The patient had a lumbar drain placed for 3 days postoperatively given the unfavorable tissue healing thought to be present within the radiated field. He was discharged on postoperative day 5 and has had no recurrence of his CSF leak after 3 months of follow-up.

Materials and Methods

Our study protocol was approved by the review board for human research at our institution. Commercial reagents and instruments, approved by the United States Food and Drug Administration for diagnostic testing and validated previously in our laboratory, were used in compliance with the instructions from the respective manufacturers, unless otherwise stated. Testing was performed according to the standard procedures and quality assurance policies of the clinical chemistry laboratory of the Department of Pathology and Laboratory Medicine at our institution.
SAMPLES
Serum, CSF, and postmortem ocular fluid samples were obtained from the clinical laboratory when surplus was available after the physician-ordered testing was completed. Tears, nasal drainage, and saliva were obtained from healthy volunteers. We used CSF and serum as positive and negative controls, respectively, on every IFE gel. CSF samples were chosen if the total protein was within the reference interval, 0.15–0.45 g/L (corresponding to 0.007–0.27 g/L total trf). The negative control was a serum sample diluted to have total protein of 0.4 g/L (total trf, 0.014 g/L).

TRF AND TOTAL PROTEIN CONCENTRATIONS
Trf concentrations were measured with commercial serum and urine assays on a nephelometer (IMMAGE; Beckman Coulter). CSF trf was determined with the urine trf assay optimized to measure trf within the range 0.002–0.04 g/L, which is compatible with the trf concentrations expected in normal CSF. Total protein was measured on the Synchro LX20 Chemistry Analyzer System (Beckman Coulter) by the biuret reaction for serum or the pyrogallol red reaction for CSF. In preliminary experiments with the Helena IFE gels, a CSF sample, and two different anti-human trf antiserum reagents, one from Beckman Coulter and the other from The Binding Site (San Diego), we observed more intense staining for the trf bands with the Beckman Coulter reagent and continued to use this antiserum in subsequent studies.

IFE PROCEDURE
IFE on the SPIFE 3000 system was performed with the IFE3/6 reagents (high-resolution agarose gels, pH 8.0). In addition to the gels and application blades, the reagent set includes packets of powdered acid violet stain, citric acid, and Tris-buffered saline, which were prepared in solutions for the IFE steps.

A summary of our procedure follows. The preprogrammed settings we use to control the electrophoresis steps are given in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue2/. Briefly, two serrated application blades are placed in parallel slots on the automated blade carrier (sled). One blade is used to apply the controls and the patient’s fluid eight times in the first row, and the second blade is used to apply the patient’s serum, if available, in a parallel row. A 15- to 17-µL aliquot of the sample (the minimum is obligatory for uniform, multiple sample loads) is dispensed into an individual well of the multisample tray. After the first sample load and application, just before the blades begin to descend into the sample tray for the next loading, the serum applicator blade is removed manually from the sled. (The serum sample is applied once.) The first blade is not disturbed and continues to make the programmed number of additional loadings and applications of the fluid sample. Each repeated application is superimposed at exactly the same site on the gel. At the end of the electrophoresis steps, 50 µL of anti-human trf (Beckman Coulter) is dispensed manually through the narrow slots in a template that is aligned over the electrophoresis lanes. After the antiserum is absorbed during the reaction with the antigen in the sample, the gel is blotted manually and dried automatically. The dried gel is transferred to the staining module of the SPIFE system for automatically controlled washing, staining, destaining, and drying. The entire procedure is completed in ~2.5 h.

NEURAMINIDASE-TREATED SERUM
A serum sample (~100 µL) known to contain a normal concentration of total trf was treated with 0.1 U of neuraminidase (Sigma-Aldrich) for 90 min at 37 °C. Such sample preparations were used as the positive controls for β-2 trf in our qualitative and quantitative studies, similar to those described in an earlier report (11).

MIGRATION REPRODUCIBILITY
The distance (mm) between the β-1 and β-2 trf bands and the distance from the cathodal end of the gel to the β-1 trf band were measured for reproducibility on 15 stained gels (5 different lots of IFE3/6 reagent sets) over a period of 11 months.

SAMPLE CONCENTRATION
We evaluated the sensitivity of trf detected in multiple superimposed applications of an unconcentrated CSF sample compared with bands seen after a single application of the same concentrated sample. Amicon Minicon-CS15 concentrators (Millipore Corp.) were used to concentrate the CSF samples.

RESULTS
A representative finished gel with samples containing β-1 and β-2 trf bands is shown in Fig. 1A. The mean (SD) distance between the β-1 and β-2 trf bands in the positive control was 2.52 (0.6) mm (n = 15; CV = 2.4%); the distance between the β-1 trf and the cathodal margin of the gel was 17.3 (0.29) mm (n = 15; CV = 1.7%).

SENSITIVITY AND SPECIFICITY
The lowest concentration of trf detected on the gel was assessed visually with serial dilutions of neuraminidase-treated serum assayed for trf on the nephelometer. Fig. 1B shows one of the sample dilutions contained a faintly stained band for which the trf result was ~0.002 g/L, the detection limit of the nephelometer. In lower dilutions having trf at higher concentrations, e.g., at 0.003 and 0.007 g/L, the trf bands were easily seen; thus, we established 0.002 g/L as the detection limit on IFE, although it is probably at a somewhat lower concentration. Among the samples other than CSF that we tested, only the ocular fluids revealed β-2 trf; tears, saliva, and nasal drainage samples from volunteers did not.
We compared the detection of trf bands on IFE after superimposing eight applications of an unconcentrated CSF sample with the same sample concentrated approximately eightfold and applied once. Total trf concentrations measured by nephelometry in the unconcentrated and the concentrated samples were 0.023 and 0.19 g/L, respectively, indicating that the concentrating process in the Minicon CS15 had occurred as estimated. Visual assessment indicated that the stained bands in the concentrated and the unconcentrated samples had similar intensities (Fig. 1C) and demonstrated that the concentration step could be replaced by eight applications of undiluted sample without a loss of sensitivity. We did not

**VERIFICATION OF MULTIPLE APPLICATIONS**

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attempt more than eight applications of unconcentrated CSF, but we did try fewer (three and six) applications, which yielded correspondingly less visualization of the trf bands.

INTERLABORATORY CONCORDANCE

Of the small number of clinical samples ordered for testing during the period of our evaluation studies, seven samples were available in sufficient surplus for us to test after the required volume was forwarded to the reference laboratory. Between-laboratory results were concordant: two were negative and five were positive for β-2 trf.

**Discussion**

Before development of the method described in this report, patient samples received in the clinical laboratory at our institution for β-2 trf testing had to be referred to an out-of-state laboratory to await a result 4 or more days after the date of sample collection. Physicians needing a rapid report in cases of suspected CSF leakage were dissatisfied with this delay and seldom ordered the test. Thus, one of our primary aims was to respond to the need for a local method that would provide results comparable to those obtained with the method being used at the reference laboratory.

We conducted the studies for verification of method performance specifications in compliance with the Clinical Laboratory Improvement Amendments standard (18). Our work was guided, in part, by the experiments described in estimable earlier reports. A summary of the main points of the present procedure compared with the other methods cited is given in Table 2 of the online Data Supplement. Several features of the latter are reviewed briefly below.

Previously published IFE methods for β-2 trf used various support media such as modified cellulose acetate, agar, or agarose (1, 2, 4, 6, 11, 12, 14–17). Some of these methods included a variation of the immunoblotting technique (4, 15, 17) to enhance the detection of trf variants. All are manual procedures involving many labor-intensive steps and 3–6 h to complete. IFE procedures on agarose typically require that several milliliters of sample be concentrated, whereas IFE in conjunction with immunoblotting uses as little as 2–5 μL of unconcentrated sample but necessitates special supplies, reagents, antibody probes, and technical expertise, which are not readily available in many routine clinical laboratories. The latter considerations notwithstanding, the ability to test microquantities of sample is a practical advantage for both the laboratory and the sample collection processes. A limitation of IFE procedures is the prozone artifact caused by antigen excess (19), a problem that is avoided with immunoblotting. The potential for excess trf in the present procedure can be controlled by preanalysis dilutions of the sample, discussed below. Most electrophoresis methods have described the detection limit for β-2 trf in qualitative terms; two reports with quantitative information are discussed in the next paragraph.

Our automated method can detect trf at 0.002 g/L or less in unconcentrated samples. This compares favorably with the detection limit (0.0025 g/L) reported by Zaret et al. (11), obtained with concentrated fluid, and reasonably well with the detection limit of 0.001 g/L reported by Normansell et al. (15), who used 3 μL of undiluted fluid in a multistep procedure involving transfer blotting and immunostaining. With a few simple modifications of the electrophoresis program on the automated system, the analysis can be efficiently completed on a high-resolution agarose gel in ~2.5 h starting with a few microliters of sample. Thus, the sample-consuming, slow process for concentration may be eliminated without loss of the sensitivity required for low concentrations of β-2 trf. The stability of the applicator blade position ensures the exact placement of replicate applications of the sample, leading to good reproducibility of band migration.

In vitro neuraminidase treatment of serum and other samples has been described by others (11, 17) for purposes similar to ours. Neuraminidase-treated serum was an important source of desialated trf in our experiments, which we used to verify the position and identity of the β-2 trf band seen on IFE and to correlate the detection limit of that band with the measurement of the specific protein by nephelometry.

Included among the various types of samples for our validation of this procedure were saliva, nasal drainage, tears, and ocular fluid. Among these, we observed that only the latter contained the β-2 trf isoform, as reported by Tripathi et al. (4). It is unlikely that ocular fluid would be mistakenly collected as a CSF leakage fluid and would not be the source of a false-positive result. Although one report interestingly argues on both sides for the need to test serum as a reference in parallel with fluid from the same patient (12), most experts favor examining serum and fluid from the same patient for simultaneous comparison to identify the rare occurrence of variant trf bands (1, 12, 15).

We were careful to characterize our procedure regarding antigen excess, a prozone artifact that can occur with IFE (19). When excessive blood is admixed in the suspected CSF fluid, the potential for this error increases. If β-1 trf exceeds the reactivity of the anti-trf antiserum, the antigen–antibody complex formed is not completely precipitated in the gel. A clear zone, representing dissolution of the immune complexes, surrounded by the precipitated protein complex is observed after drying and staining of the gel. To reduce the risk of excess β-1 trf antigen, we routinely measure total protein in the patient sample as a guide for whether to dilute the sample. If the total protein is >1.25 g/L, we prepare serial dilutions of the sample and perform IFE on the undiluted sample and the dilutions, which allows efficient detection of β-2 trf, if present.

We can recognize antigen excess by the appearance of two
curved bands at the edges of an inner colorless zone indicating the excess antigen. It is important to differentiate this pair of bands from the two separate isoforms (Fig. 1D). We have noted three features that help identify \( \beta-2 \) trf if it is present with \( \beta-1 \) trf: (a) the \( \beta-1 \) band is dominant; (b) the two isoform bands are separated by a predictable and reproducible distance; and (c) the two isoform bands are parallel and straight, not curved.

We anticipated that the source of antiserum would be important. Zaret et al. \( \text{(11)} \) reported that multiple manual applications of undiluted samples on high-resolution agarose by one system combined with use of a potent antiserum yielded results for \( \beta-2 \) trf that were superior to those obtained with another antiserum and a different IFE agarose reagent set and system. Our experience was similar with respect to the quality of antiserum reagents, i.e., the Beckman Coulter product we preferred produced trf bands that were more intensely stained than bands obtained with antiserum from another supplier. We interpreted this as indicating that the preferred anti-trf antiserum had a higher titer, avidity, affinity, and/or specificity for human trf. The stains and colorizing reactions most often described for visualization of \( \beta-2 \) trf after electrophoresis include Coomassie blue \( \text{(11, 14)} \), silver \( \text{(5, 12)} \), and immunochemical probes \( \text{(4, 15, 17)} \). The latter involve enzyme-labeled conjugates together with transfer blotting to confer enhanced sensitivity. Acid violet stain is included with the Helena IFE assay because the manufacturer found it to be more sensitive than other stains for the detection of various normal and abnormal proteins (Dr. Rita Ellerbrook, Helena Laboratories, personal communication, letter dated August 6, 2004). After we found that the detection limit was satisfactory with the manufacturer’s method of staining, we did not to modify this part of the automated program, thereby minimizing the number of manual interventions in the procedure.

Although the number of samples in our interlaboratory study was small, we were encouraged by the complete concordance because our results were obtained with unconcentrated samples whereas the results reported by the other laboratory were based on a different IFE procedure using concentrated samples. The interlaboratory agreement of results completed our validation studies.

In addition to the case report above, study of another in our series of patients demonstrated the clinical utility of the method we have described. This patient had suffered a septal fracture in a motor vehicle accident and subsequently complained of clear rhinorrhea. Samples of her rhinorrhea fluid over a period of several months have contained no detectable \( \beta-2 \) trf. She is currently being managed for vasomotor rhinitis with no definitive evidence for CSF leakage and has avoided further invasive testing.

In the most recent 12 months since the inception of this method for routine use in our institution, \( \beta-2 \) trf testing was ordered in 48 samples. For 46 (96\%) of these samples, the volume was \( \geq 15 \mu L \), sufficient for testing, whereas 2 nasal samples could not be tested because they were not usable (dried mucus).

Faced with a diminishing, less-experienced staff and more funding constraints in recent years, we took advantage of new automation and developed a well-characterized procedure that yields reproducible and sensitive results comparable to those obtained by more labor-intensive procedures, which are not feasible in the current laboratory environment. An interpretive statement accompanies the qualitative result to advise the physician of the detection limit of the method. The combined advantages of small sample volume, good sensitivity, and rapid results have created a threefold increase over the 13 requests received in the 12 previous months when testing was referred to an out-of-state laboratory. The increased use supports our conclusions that this method provides the physicians at our institution with a rapid, reliable, noninvasive aid in the diagnosis and management of patients with suspected CSF leakage.

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**References**