A Rapid Silver-Stain Procedure for Use with Routine Electrophoresis of Cerebrospinal Fluid on Agarose Gels

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Oligoclonal banding patterns, when present in cerebrospinal fluid (CSF) electrophoresed on agarose gels, are important in the diagnosis of multiple sclerosis and other demyelinating diseases. Typically, an 80-fold concentration of 2.5 mL of CSF has been needed before the oligoclonal banding pattern could be detected with Coomassie Brilliant Blue stain. This excessive volume requirement often is a problem, particularly in children. We describe here an improved silver-stain procedure with which we routinely can detect the oligoclonal banding pattern in 1 to 3 mL of unconcentrated CSF. The procedure is inexpensive and takes less than 45 min. Our method is quick, safe, sensitive, and easily performed in the clinical laboratory.

Additional Keyphrases: oligoclonal bands • pediatric chemistry • multiple sclerosis • neurological disorders

Multiple sclerosis (MS) is among the more common demyelinating diseases, afflicting approximately 100,000 people in the United States (1). Its etiology is unknown, but most of the central nervous system manifestations evidently result from a loss of nerve conduction, owing to breakdown of the myelin sheath (1). Both autoimmune and genetic mechanisms have been implicated; frequently, exposure to viral antigens (especially measles) or a T-lymphocyte abnormality, or both, have been described (1, 2). Symptoms usually first appear in the third to fifth decades, associated with various neurological deficits: blurred vision, lack of coordination, paresthesias. In addition to these clinical features, function tests such as evoked potentials and the laboratory analysis of cerebrospinal fluid (CSF) are used to confirm the diagnosis (3). Currently, abnormal values for the CSF IgG Index and for CSF electrophoresis are the most sensitive and specific laboratory findings: more than 90% of definite MS patients demonstrate abnormalities in one or both (4, 5).

Both the IgG Index and CSF electrophoresis are based on the observation that intrathecal synthesis of IgG is increased in MS and other demyelinating disorders. More specifically, the intrathecal IgG has been observed to migrate as distinct bands in the slow-beta and gamma electrophoretic regions (6), a pattern referred to as "oligoclonal banding," and these discrete bands are believed to represent production by individual clones of lymphocytes in the central nervous system (7). Oligoclonal banding usually is demonstrated by zone electrophoresis or isolectric focusing on polyacrylamide or agarose (8, 9). Because the protein content of CSF is so low (usually <600 mg/L, only 20% of which is IgG), an 80- to 100-fold preconcentration of CSF is required if the oligoclonal bands are to be visible when Coomassie Brilliant Blue R-250 is used as the protein stain. Frequently, 2.5 mL of CSF is needed, which presents numerous difficulties, including lack of analysis because of insufficient sample volume.

The sensitivity of the silver-stain procedure, about 50- to 100-fold that for staining with Coomassie Blue, has revolutionized detection of proteins present in body fluids at low concentrations (10, 11), CSF electrophoresis being a prime example. However, silver-stain procedures designed for use with polyacrylamide do not work with agarose gels (12, 13). For various reasons, polyacrylamide is not widely used in clinical laboratories, but agarose has gained popularity for routine use in both protein and isoenzyme electrophoresis. Our adaptation of the silver-stain procedure to agarose solves the following problems with earlier published procedures: (a) lack of sensitivity and reproducibility, (b) high backgrounds with "grainy" staining, or (c) time-consuming pretreatments of the agarose gels with toxic chemicals (14-20). The procedure we describe here represents a marked improvement in analysis time (total time from start of electrophoresis to stained gel is less than 2.5 h), sensitivity, cost, and elimination of toxic chemicals.

Materials and Methods

Agarose plates from Beckman (SPE II Gels; Beckman Instruments Inc., Brea, CA 92621) and Helena (Titan; Helena, Inc., Beaumont, TX 77704) are easily stained by our method. Tungstosilicic acid was obtained from BDH Chemicals Ltd., Poole, U.K. Other reagents were purchased from Fisher Scientific, as were the weighing boats and plastic forceps. Distilled water was used throughout; Styrofoam cups were purchased at a local food store.

Electrophoresis and gel manipulation. Gels were electrophoresed according to manufacturers' instructions, fixed for 5 min in methanol/water/acetic acid (5/5/1 by vol), and dried for 20 min in a forced air oven at 65°C. At this point the gel could be either stored or stained immediately. To avoid fingerprints on the gels, we used plastic forceps to transfer the gel during this entire procedure. Gels were stained in weighing boats placed on a platform shaker. More than one gel may be processed at a time without increasing the reagent volumes. Solutions are stable for at least one month.

Staining. Place the gel in 100 mL of a 25 g/L solution of glutaraldehyde for 10 min. Transfer the gel to another boat containing 100 mL of a 5 mL/L solution of Triton X-100 for 5 min; repeat this step once.

Now transfer the gel to another boat containing 100 mL of a 0.1 g/L dithiothreitol solution to soak for 10 min. During this time, prepare the silver solution as follows:

(a) Add 47 mL of water to a new Styrofoam (aerated polystyrene) cup, which has been rinsed with distilled water. The cup also contains a magnetic stirrer bar, which has been washed with concentrated HNO3 and rinsed with water.

(b) Add 1 mL of AgNO3 (100 g/L), while stirring.

(c) Add 1 mL of NH4NO3 (120 g/L).

(d) Add 1 mL of tungstosilicic acid (500 g/L).

(e) After mixing for 30 s, add 0.7 mL of 370 g/L formaldehyde solution.

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For convenience, larger volumes of this silver solution may be made up and stored at 4 °C for at least a month.

To another new Styrofoam cup containing an acid-washed, water-rinsed magnetic stirrer bar, add 50 mL of 50 g/L Na₂CO₃ solution. While rapidly stirring, gradually (during 10 s) add the silver solution to the Na₂CO₃ solution. Immediately pour this developing solution into a weighing boat. Be sure no particulates are present. Remake the solution if it is not clear—the most common cause of precipitate formation is lack of cleanliness.

Now transfer the gel to the developing solution and watch for the appearance of protein bands within 3 to 5 min. A fine, brown precipitate should also begin to appear slowly; eventually this darkens to a black precipitate. It is best to remove the gel within 5 min to avoid the grainless and high-background staining that result from the deposition of the dark precipitate.

When the pattern is sufficiently dark, or when the precipitate has formed, arrest the development by placing the gel in fixing solution for 1 min. Then dry the gel for 5 min in a forced-air oven for a permanent record. The dried gel may be placed into fresh developing solution if the protein bands are insufficiently dark (N.B.: at this point the staining reaction occurs very rapidly and must be closely monitored). The most sensitive results with the lowest backgrounds are usually obtained with this double-stain procedure. The dried gel image is stable and can be used as a permanent record of the electrophoresis.

**Results**

Silver staining is much more sensitive method for detecting proteins than the Coomassie Brilliant Blue procedure. Figure 1 (A to C) shows a typical protein pattern obtained by using Coomassie Brilliant Blue with an undiluted serum sample and with a CSF sample concentrated 80-fold. Other portions of these same samples were also stained with the silver stain; however, the serum was diluted 100-fold and the CSF sample was un-concentrated (Figure 1, D to F). The sensitivity of the silver stain is known to vary with the specific protein (12, 13). However, as can be seen, the silver stain and the Coomassie stain produce similar results, which is typical of our experience with serum and CSF.

Figure 2 illustrates results for an assortment of unconcentrated CSF samples, some from patients with demyelinating diseases and oligoclonal bands and some from patients without obvious demyelination or bands. Clearly, the low protein concentrations in the oligoclonal bands are easily distinguished by use of the silver stain. Electrophoresis of serial dilutions of protein standards demonstrates that the sensitivity of the silver stain in detecting proteins is in the range of 5 ng (data not shown).

**Discussion**

The exact mechanism of the silver stain in agarose and polyacrylamide gels is not known, but it is believed to involve an amplification process whereby silver is deposited autocatalytically on an initial nucleation site (12, 21, 22). This site is thought to be a complex between silver ions and amino acid sidechains of proteins—probably the sulphydryl, amino, or carboxyl groups (23, 24). Differences between the agarose and polyacrylamide procedures are ascribable to differences in the matrices. The polyacrylamide gel matrix, which lacks these nucleation sites, can be silver-stained without a drying step (10, 25). Interestingly, an undried agarose matrix is unsuitable for silver staining (12, 13); the conformation of the hydrated agarose appears to expose so many nucleation sites that background staining is high. Drying the agarose gel after fixation apparently decreases the number of available nucleation sites in the agarose matrix, thus keeping the background low. Additionally, several modifications of the polyacrylamide gel procedure are prerequisites for using it with agarose. For instance, an oxidant and mordant, tungstosilicic acid, must be included in the developing solution, to make silver staining more reproducible (14, 15, 21). This chemical prevents silver ions from being reduced immediately by formaldehyde, precipi-

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**Fig. 1. Coomassie Blue stain and silver stain compared**

Samples were electrophoresed 40 min at 100 V on a Beckman SPE-II agarose gel. Lanes A–C: stained with Coomassie Blue R-250. Lanes D–F: silver-stained. A, 2 μL of a control mixture of myeloma patients' sera; B, 1 μL of serum from a multiple-sclerosis patient; C, 1 μL of CSF from a multiple-sclerosis patient, concentrated 80-fold (note oligoclonal bands); D, 2 μL of a control mixture (same as Lane A) diluted 20-fold; E, 2 μL of serum (same as Lane B) diluted 100-fold; F, 2 μL of unconcentrated CSF (same as Lane C) (note oligoclonal bands).

**Fig. 2. Silver stain of unconcentrated CSF**

Samples were stored at 4 °C and electrophoresed as in Fig. 1. To ensure uniform staining, we electrophoresed either 1 or 3 μL of unconcentrated CSF, depending upon prior knowledge of protein concentration. Lanes A, C, and E: unconcentrated CSF with a normal IgG index and no oligoclonal bands. Lanes B, D, and F: unconcentrated CSF with an abnormally high IgG index and an oligoclonal banding pattern.
tating out of solution, and depositing in the agarose gel before the silver can preferentially stain the protein. In contrast, when the first reliable silver-stain procedure was introduced in 1979 for polyacrylamide gels, tungstosilicic acid was not needed (10); unfortunately, that procedure does not work well for agarose gels.

Several improvements make it possible to silver-stain agarose gels in the clinical laboratory (12, 13, 17–20). The method described here draws from several of these and adds some further refinements. All methods involve the same developing solution, with minor variations in ratios. The developing solution contains Na₂CO₃, AgNO₃, NH₄NO₃, formaldehyde, and the "magic ingredient," tungstosilicic acid. One improvement we have made is to do double-stain, with a short drying step in between; by so placing the agarose matrix into a non-silver-binding conformation, background is lower and the gel has a less grainy appearance. This is important because it allows for much longer staining, with resulting increased sensitivity.

Other differences between this and earlier procedures lie in the fixing and pre-staining steps. In previous methods, these are either very long, are unnecessarily complicated, or involve the use of potentially toxic chemicals such as potassium ferrocyanide. The glutaraldehyde and dithiothreitol pretreatment steps enhance the sensitivity of silver staining in both polyacrylamide gels (23, 25) and agarose gels (26), contrary to reports by others (12). One additional pretreatment, the wash step with Triton X-100, decreases staining time and decreases the mottled, gray-black, splotchy appearance that occasionally appears (personal communication, Chandravadan Patel, Helena Laboratories). Triton X-100 detergent can act as a surfactant to help "wet" the gel; its mechanism of action is not clear, but it may be affecting the conformation of the agarose matrix and masking potential silver nucleation sites.

Although the advantage of using unconcentrated CSF makes the silver stain very attractive, there are some disadvantages. Silver staining of unconcentrated CSF takes longer than Coomassie Blue staining of concentrated CSF, but this is offset by eliminating the several hours needed to concentrate CSF. Extreme attention to cleanliness is mandatory; for instance, fingerprints will be stained by this extremely sensitive procedure, so plastic tongs are used to handle the gels. Any apparatus that is re-used, such as the magnetic stirrer bars, must be washed with concentrated nitric acid. Use of inexpensive, disposable Styrofoam cups and weighing boats allows us to avoid acid-washing glassware.

Compared with other published methods, our method is quicker, safer, and more easily performed in the clinical laboratory. It also is versatile and inexpensive. We have tried the procedure on Beckman and Helena agarose gels and on our own homemade agarose gels, with equally good results. Current cost of the stain reagents necessary to stain one gel is less than a dollar.

Silver-staining of agarose gels also has great potential in the examination of other dilute body fluids, such as urinary proteins for the determination of kidney abnormalities (4), prostatic fluid, synovial fluid, and saliva (4, 19). The silver stain has in effect given us 100- to 1000-fold more body fluid protein to analyze and should be of use in future electrophoretic examinations in the clinical laboratory.

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