

Quantitative Detection of Type A Staphylococcal Enterotoxin by Laurell Electroimmunodiffusion

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The detection of staphylococcal enterotoxin A by the quantitative technique of electroimmunodiffusion is described. High dilutions of type-specific rabbit antiserum were used in 1% agarose gels, 1 mm thick, and prepared in 0.05- μ g barbital buffer, pH 8.6. Volumes of 10 μ liters containing 1.5 to 10 ng of toxin were electrophoresed out of 4-mm diameter wells at 5 mA/cm width of gel. The precipitin cones formed were made visible by first immersing the agarose gels in 0.2 M NaCl and then overlaying the surface with the purified globulin fraction of sheep serum against rabbit globulin, followed by soaking of the gels in 1% aqueous cadmium acetate and staining with 0.1% thiazine red in 1% glacial acetic acid. Fully extended cones, 4 to 23 mm in length depending on toxin concentration and antiserum dilution, were developed in 2 to 5 h of electrophoresis, and visualization was achieved within 2 to 3 h. Because the method is qualitative, quantitative, simple, rapid, and sensitive, it offers a practical tool for the detection of small amounts of bacterial toxins in contaminated foods. The method should also qualify as a sensitive detection device in biochemical procedures which attempt to trace, detect, and identify biological substances in nanogram quantities, provided these substances are antigenic and capable of forming a precipitate with their specific antibodies.

The term electroimmunodiffusion has been used to describe a sensitive tool for the quantitative estimation of proteins in solution. The technique combines the two separate processes of conventional immunoelectrophoresis, electrophoresis and immunodiffusion, into a single step: the antigen is forced by an electric field into a gel which already contains the corresponding antibody. The antigen moves in a linear fashion until titrated to equivalence with the antibody. The resulting precipitin pattern assumes the shape of a cone whose tip is rounded when there is still an excess of antigen present and pointed when all antigen has been precipitated. For a given antibody dilution and constant electric field, the length of the precipitin cone is proportional to the antigen concentration.

The technique was introduced by Laurell (7) and has been used almost exclusively as a tool for serum protein analyses in clinical medicine. Studies by Lopez et al. (8) indicated that, in

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principle, it should be possible to utilize electroimmunodiffusion for the detection and identification of any protein soluble in electrolyte solutions of low ionicity. Miller and Anderson (10) were able to measure and identify as to type very small quantities of *Clostridium botulinum* toxin. Preliminary investigations of the antigenic components of *Aeromonas salmonicida*, a pathogen of salmon and trout, have shown that the technique is able to detect cell wall lipopolysaccharides as well (L. Udey, personal communication). Most recently, the two-dimensional version of the technique was used by Roberts et al. (12) to characterize mycobacterial antigens.

The present study was undertaken to develop a practical methodology for the detection of small amounts of bacterial toxins in contaminated foods. In this country, staphylococcal contamination accounts for 25 to 30% of all reported food-borne outbreaks and in these type A is the most frequently encountered toxin. Many techniques are available for the detection of staphylococcal enterotoxins as re-

viewed in detail by Bergdoll (1). Since the review, a rapid capillary tube assay has been described (4, 5). At the present time, however, the Casman microslide diffusion assay (3) is the practical method of choice for detecting small amounts of enterotoxin, 0.1 to 1.0 $\mu\text{g}/\text{ml}$, in concentrated food extracts. The technique requires a four-step procedure which takes 5 to 7 days for completion and close control over the antigen-antibody ratio (1, 9).

This report describes electroimmunodiffusion as a method whose sensitivity is well within the range of the microslide technique and, in addition, offers the distinct advantages of speed and simplicity. Furthermore, the time span between the extraction of the toxin from foods, its concentration, and application to the antibody-containing gel for electrophoresis may be shortened considerably because the toxin no longer needs to be as purified as for the microslide assay. The projected usefulness of the technique in the detection of enterotoxins from foods is being investigated in our laboratories.

MATERIALS AND METHODS

Buffer. A barbital-NaOH buffer of 0.1 μ ionic strength and pH 8.6 was prepared with deionized distilled water by the method of Campbell et al. (2). Buffer of 0.05 μ was prepared by diluting the 0.1- μ buffer with an equal volume of deionized distilled water.

Antigen. The purified staphylococcal enterotoxin A used in this investigation was given to us by M. S. Bergdoll of the Food Research Institute and Department of Food Science, University of Wisconsin. The enterotoxin was supplied in lyophilized form and reconstituted in 0.37% Brain Heart Infusion broth as suggested by Bergdoll. This stock solution was stored at -20°C . Working solutions for experimentation were prepared by 10-fold serial dilutions in 0.05- μ buffer preserved with 0.1 mg of merthiolate per ml. These solutions were stored in small screw-capped vials at 4°C . Typical concentrations were 100 μg of toxin protein per ml, 10 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$, and 0.1 $\mu\text{g}/\text{ml}$. No loss of antigenicity was noted over a period of at least 2 months as judged by comparison with working solutions freshly prepared from the frozen stock solution.

Antibody. The lyophilized rabbit antiserum, also supplied by M. S. Bergdoll, was restored to its original volume by adding 1.0 ml of 0.05- μ buffer preserved with 0.1 mg of merthiolate per ml. The working solution was prepared by diluting 1:4 with the same buffer. Storage was at 4°C . Optimal sensitivity of the freshly reconstituted antiserum was not attained until after 7 to 10 days of standing at the storage temperature (the reason is as yet unknown). An antiserum dilution of 1:36 would detect 1 μg of toxin protein per ml by using the Casman microslide technique.

Preparation of antibody-containing agarose gels. The solid support medium consisted of 1% agarose in 0.05- μ barbital buffer, pH 8.6. For reasons of convenience and reproducibility, several hundred milliliters of the agarose support were prepared by boiling in the buffer until melted, cleaned of foreign matter by filtration through fritted glass (coarse), dispensed in measured quantities into screw-capped vials, and stored at 4°C . When needed, the content of a vial was remelted in boiling water and equilibrated to 47°C in a constant temperature water bath. Antiserum from the working solution was then added (vol/vol) to give a desired final concentration. The vial was gently rolled between the palms of the hands or tilted back and forth to assure uniform distribution of the enterotoxin antiserum in the agarose. By using a warmed pipette, the antibody-containing agarose was poured into a preformed mold and left to solidify for 10 to 15 min at 4°C .

Preparation of mold. The agarose frame consisted of a plastic template (81 by 100 by 1 mm) sandwiched between two photographic glass slides (81- by 100-mm lantern slide), all held together by several strong clamps. A section (50 by 70 mm), cut out of the plastic template, provided the space for the melted agarose (the 70-mm edge ran parallel to the 81-mm edge of the glass slides). The glass slides were cleaned by autoclaving or steaming in a mild detergent solution for about 15 min, thoroughly rinsed in distilled water, and stored in Photo-Flo 200 solution (Eastman Kodak, Rochester, N.Y.) until needed. When a mold was assembled, the slides were again rinsed in distilled water. The clamps were arranged such that the frame could be placed upright on a level surface so that one could pipet the agarose from the top, carefully avoiding to entrap air bubbles.

Preparation of agarose gel for electrophoresis. After hardening of the gel, the clamps were removed and the remainder of the mold was placed on a level surface so that the closed edge of the template rested against a stop plate. The top glass slide was then carefully pushed off the gel and over the stop plate. The plastic template was peeled away also, and any excess moisture around the slab of antibody-containing agarose was removed with disposable wipers. The center section (40 by 50 mm) of the gel was covered with a Lucite plate, leaving on either side of it sections (15 by 50 mm) of gel for contact with electrical bridges. The Lucite plate had overall dimensions of 40 by 100 by 4 mm, with a 40 by 10 by 1 mm support base left at either end. The height of the support base thus matched the thickness of the agarose gel so that the Lucite cover could be placed across the gel without exerting undue pressure. It was essential (i) to exclude any air bubbles from the interface between the gel and the Lucite cover plate (this was easily accomplished by placing a few drops of 0.05- μ buffer on the proper face of the cover plate, inverting it, and then slowly lowering it into position on the gel slab), and (ii) to remove any excess liquid along the outside edges (70 mm) of the gel. Four sample wells with a diameter of 4 mm each were cut into the gel with a sharp-edged glass tube by reaching through 6-mm diameter holes drilled into

the Lucite cover plate. These holes were centered 10 mm apart on a line 5 mm away from and parallel to the edge of the plate (Fig. 1).

Electrophoresis. The glass slide with the prepared gel was then placed on a cooling surface in a conventional immunoelectrophoresis unit. Cooling was provided from a 24 C constant temperature water bath. Both electrode vessels contained 0.1- μ barbital buffer, pH 8.6. The electrical bridges were constructed from strips (55 by 70 mm) of Whatman no. 3MM filter paper. The wicks were moistened in the buffer, and the short edges were placed flush against the Lucite cover plate. Thus, no part of the gel surface was exposed to the air. To avoid sharp bending of the wicks at the edges of the glass slide, the wicks were draped over plastic shoulder strips as commonly used in immunoelectrophoresis. Finally, the entire surface of the wicks exposed to the air was covered with a plastic film.

A constant current of 25 mA (5 mA/cm) was applied for 5 to 10 min until the voltage had dropped to a constant value. This value was then adjusted to 10 to 11 V/cm (as measured between the wicks) by lowering or raising the buffer level in the electrode vessels. Once adjusted, this voltage level was always attained in subsequent runs. After the electrophoretic conditions had settled to constancy, the sample wells were filled with 10- μ l volumes which contained varying quantities of staphylococcal enterotoxin A. Electrophoresis lasted from 1 to 8 h. The life of the buffer was extended by switching the polarity on the electrophoresis unit after the completion of each experiment (the row of sample wells in the agarose gel was always on the side of the cathode).

Detection of precipitin cones. After electrophoresis, the electrical bridges and the Lucite cover plate were removed and the agarose gel was immersed in 0.2 M NaCl for 1 h at room temperature and then rinsed briefly in distilled water. Any excess water was removed by carefully touching absorbent wipers to the sides and the surface of the gel. The surface was then overlaid with sheep anti-rabbit globulin (purified globulin fraction, Nutritional Biochemicals Corp., Cleveland, Ohio) diluted 1:10 in 0.05- μ barbital, pH 8.6, and preserved with 0.1 mg of merthiolate per ml. After 1 h of reaction time at room temperature, most of the globulin was recovered and stored at 4 C to be reused. The remainder was rinsed off under a gentle stream of distilled water followed by immersion of the gel slab in a 1% aqueous solution of cadmium acetate. Overlaying the agarose-antiserum gel with sheep anti-rabbit globulin and immersing it in cadmium acetate increased the size and density of the original antigen-antibody complexes and thus enhanced their visibility. The precipitin cones were seen to develop fully within 15 to 30 min. Staining for 15 to 30 min with 0.1% thiazine red dissolved in 1% acetic acid enhanced the visibility of the cones still further. The precipitin lines could be observed best by placing the gel on a dark-field viewer box fitted with a circular fluorescent light source as described by Williams and Chase (14). The length of the precipitin cones was measured to the

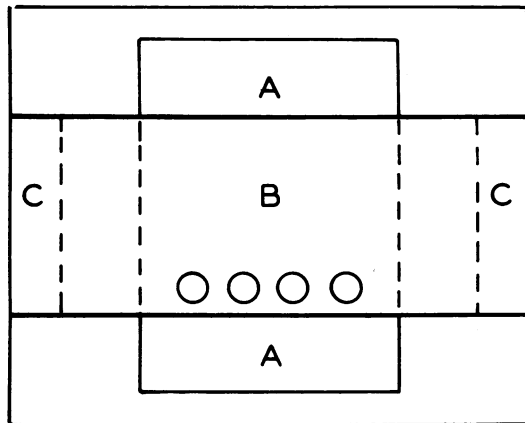


FIG. 1. Schematic of agarose gel and Lucite cover plate resting on photographic glass slide. A, Gel contact areas for electrical bridge connections; B, Lucite cover plate provided with four holes for sample wells; C, support bases of Lucite cover plate.

nearest 0.5 mm from the center of the sample well to the tip.

Records. The agarose gel, transferred onto a thoroughly cleaned lantern slide, was immersed in 95% ethyl alcohol for 1 h at room temperature. After draining off the excess alcohol, the gel was dried to a thin film at 37 C, properly labeled, and stored in a dry, dust-free box. The treatment with alcohol assured rapid and undistorted drying of the gel. When a slide needed to be reviewed, the dried gel had to be wetted first before the precipitin cones became visible.

A standard Polaroid MP-3 industrial view camera, the dark-field viewer box, 4 by 5 contrast process ortho film 4154 (Eastman Kodak), and Agfa Brovira BEH1 grade 6 printing paper were found to be most useful for making photographic records.

RESULTS

Detection of enterotoxin. Figure 2 shows a representative example of the appearance of the precipitin cones after electrophoresis of the enterotoxin A into the antibody-containing agarose gel. The rounded ends of the two longer cones on the right indicate antigen excess. Further electrophoresis lengthened these cones and stretched their tops into sharp end points. The two smaller cones on the left have already reached their optimal lengths. Electrophoresis of only 1-h duration produced cones with rounded ends and precipitin lines which were rather broad and diffuse and perpendicular to the diameter of the sample well.

Figure 3 demonstrates the dependence of cone length on the antibody dilution in the agarose gel: for any of the tested toxin concentrations the cone length increased as the antibody concentration decreased. The smallest

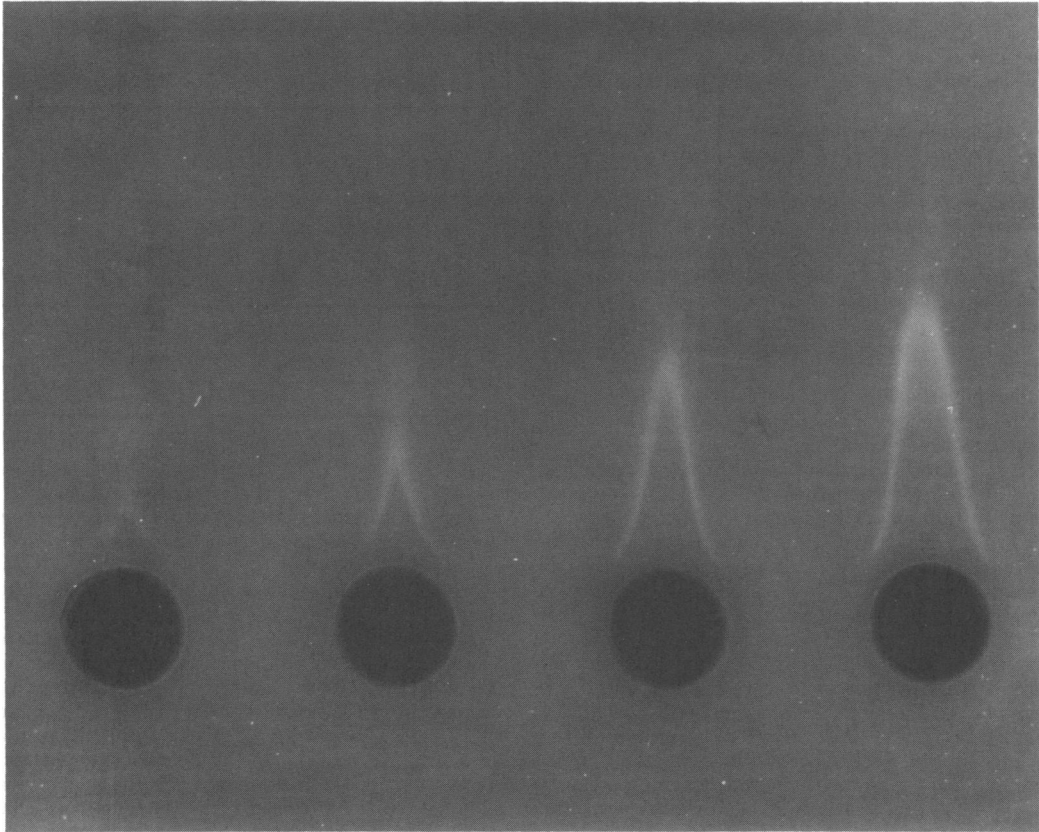


FIG. 2. Appearance of the precipitin cones after 3 h of electrophoresis. Enterotoxin A concentrations, from left to right: 0.15, 0.30, 0.60, and 1.0 $\mu\text{g}/\text{ml}$ (corresponding to 1.5, 3.0, 6.0, and 10 ng of toxin per well, respectively). The antiserum dilution was 1:200 in the agarose gel.

quantity of toxin that could be visualized reliably was 0.15 $\mu\text{g}/\text{ml}$, representing only 1.5 ng of protein applied to the sample well. The corresponding optimal cone length varied from 4 mm at the 1:200 dilution of the antiserum in the agarose (Fig. 3A) to a length of 7.5 mm at the 1:400 antibody dilution (Fig. 3C). The time of electrophoresis necessary to reach these lengths increased from 1 to 3 h, respectively. The largest quantity of toxin tested, 10 ng (1.0 $\mu\text{g}/\text{ml}$), produced an optimal cone length of 13.5 mm at the 1:200 dilution and 23 mm at 1:400, either length requiring 5 h of electrophoresis for completion. The precipitin lines decreased in intensity as the antiserum dilution increased. The limit of visibility was reached at a 1:480 antibody dilution (not shown in Fig. 3).

Electrophoresis. Although agarose is a highly purified form of agar and supposedly neutral in charge, our supply demonstrated considerable electroosmosis under the described experimental conditions. By covering

the center portion of the gel with a Lucite plate, the gel was prevented from swelling at the cathode, thereby applying a backpressure on the electroosmotic flow of water from the anode to the cathode, with the result that the agarose gel retained its even thickness. However, electroosmosis was not totally eliminated, as demonstrated by the slight migration of a neutral dye out of its cathodic well. By covering both gel and electrical bridges, any changes in the homogeneity of the electric field due to evaporation of moisture were held to a minimum.

DISCUSSION

In our attempts to find the sensitivity limit of electroimmunodiffusion as applied to the detection of staphylococcal enterotoxin A, it was found that toxin concentrations of 2 $\mu\text{g}/\text{ml}$ (20 ng/well) and higher can be detected as much more sharply defined precipitin cones than those shown in Fig. 2 when the type-specific antiserum is diluted only 1:80 to 1:100 in the agarose gel. For visualization, immersion

into 0.2 M NaCl is sufficient. Staining of the dried gels with thiazine red produces cones intense enough to be used as negatives for photography or slide projection. Further attempts to lower the detection sensitivity into the 0.1 to 1.0 $\mu\text{g/ml}$ range (1–10 ng/well), however, failed to produce visible precipitin lines. In electroimmunodiffusion these small amounts of antigen require high dilutions of antibody in the agarose gel to form precipitin cones of measurable length. However, the antigen-antibody complexes become so thinly distributed along the line of precipitation that they are no longer visible to the eye. For the detection of enterotoxin quantities of 10 ng or less it becomes necessary, therefore, to develop means of visualization. For the time being, we have found it practical to use the precipitated antigen-antibody complex as a new antigen and to allow it to react with sheep anti-rabbit globulin applied to the gel as an overlaying solution after electrophoresis. The resulting precipitin complex is enlarged even further by chelating with cadmium. This procedure, in turn, reaches its limit of reproducible detection at 1.5 ng of toxin per well and an antiserum dilution of about 1:400. The precipitin lines become markedly fainter after that, and visibility is finally lost when the antiserum is diluted about 1:480 in the agarose. A higher titered antiserum, therefore, does not provide an automatic decrease in the detection limit beyond 1.5 ng, although electroimmunodiffusion is capable of producing precipitin complexes corresponding to considerably smaller quantities of enterotoxin. The challenge, then, consists in finding practical techniques to enlarge the size of these antigen-antibody complexes until they become visible.

As far as the reproducibility of the electrophoretic conditions is concerned, one should be aware of the drawbacks inherent to agar gel electrophoresis (11, 13). We found the electric field to be fairly constant for 2 to 3 h, and then to slowly decline in strength. This decline did not always take place at the same rate. This observation in no way distracts from the fact that the optimal cone length is proportional to the enterotoxin concentration. The observation does mean, however, that the optimal length may be reached somewhat earlier or later in time than indicated in Fig. 3. From an applied point of view it is, therefore, prudent to run a standard toxin concentration together with the unknown sample and, by comparison of the cone lengths and shapes, to decide whether the unknown sample contains a higher or lower toxin concentration than the standard.

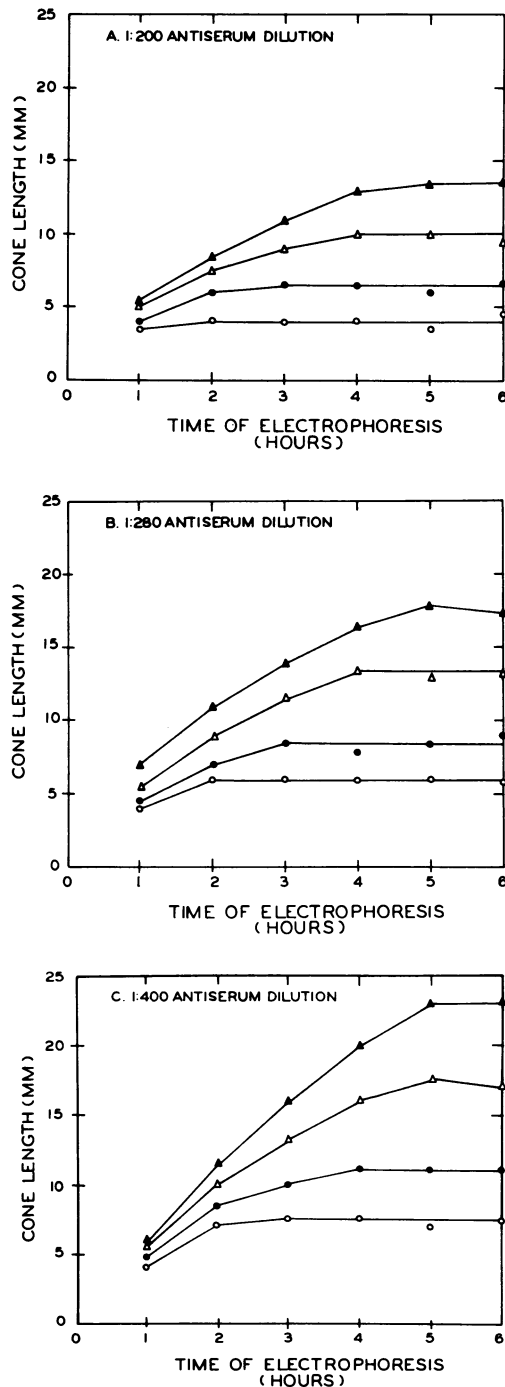


FIG. 3. Dependence of precipitin cone length on the duration of electrophoresis, enterotoxin concentration, and antibody dilution in the agarose gel. Enterotoxin A concentrations: O, 0.15 $\mu\text{g/ml}$; ●, 0.30 $\mu\text{g/ml}$; Δ , 0.60 $\mu\text{g/ml}$; \blacktriangle , 1.0 $\mu\text{g/ml}$ (corresponding to 1.5, 3.0, 6.0, and 10 ng of toxin applied to each well, respectively).

In summary, electroimmunodiffusion offers the simplicity, versatility, speed, and sensitivity expected from a practical detection technique. There is no need for a close control of the antigen-antibody ratio. The number of samples which can be tested simultaneously is limited only by the size of the electrophoresis unit and the electric field strength that can be delivered with constancy. Antigenic cross-reactivity of the toxins is not a problem because of the high concentration of heterologous toxin necessary to compete with the homologous toxin for the antibody binding sites (6). Furthermore, the pH of the electrophoresis buffer is very near the isoelectric point in the case of enterotoxin B. At present, the method just described has a detection limit of 1.5 ng of enterotoxin A. In principle, however, electroimmunodiffusion is capable of providing a much lower sensitivity; the difficulty lies in making the immune complexes visible and measurable.

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