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Investigation of a Method to Increase the Sensitivity of Counter-Current Immunoelectrophoresis Using Direct-Contact Photography

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Title of Thesis INVESTIGATION OF A METHOD TO INCREASE THE SENSITIVITY OF COUNTER-CURRENT IMMUNOELECTROPHORESIS USING DIRECT-CONTACT PHOTOGRAPHY

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INVESTIGATION OF A METHOD TO INCREASE THE SENSITIVITY OF COUNTER-CURRENT IMMUNOELECTROPHORESIS USING DIRECT-CONTACT PHOTOGRAPHY

by

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ABSTRACT

Counter-current immunoelectrophoresis (CIE) has been used to detect bacterial capsular polysaccharides in both cerebrospinal fluids and blood serum of children with systemic meningococcal diseases. The sensitivity of the method depends upon the titre of the antisera. It was found that the sensitivity of CIE could not be increased by the method of direct-contact ultraviolet photography. The sensitivity could be increased by one serial dilution, on the average, by the treatment of a 0.25% tannic acid wash for 15 minutes.

ACKNOWLEDGEMENTS

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DEDICATION

This thesis is dedicated to

Frances and Elizabeth

who understandingly accepted my absence

on many weekends and evenings,

time which was rightfully theirs.

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INTRODUCTION

For the past decade the diagnosis of bacterial meningitis by CIE procedure has become a widely accepted and useful diagnostic tool. It has been shown [1-5] to be an extremely sensitive and specific method of diagnosis, avoiding many of the problems encountered by examination of gram stain smears and cultures.

It has been found that the sensitivity of the CIE procedure depends upon the titre of the antisera used.[6] The white-colored proteins formed by the antigen/antisera reaction show density not only to light but, also, to a greater extent, to ultraviolet radiation between 280 and 200nm.[7-9] It was hoped that this inherent sensitivity of the CIE procedure could be increased by the use of direct-contact ultraviolet photography utilizing an ultraviolet source and film to record the absorption of the protein line formed by the antigen/antisera reaction. Photographic methods have been used to detect nucleic acids in gel [10-11] and proteins separated in starch gel.[12] In an attempt to increase the absorbence of the protein line formed by the antigen/antisera reaction the agarose gel was stained with amido black and tannic acid. It was found that ultraviolet photography was of no use in increasing CIE sensitivity. The treatment of the agarose gel with tannic acid did increase the sensitivity of the visually observed result without the use of ultraviolet photography. This method did not interfere with the normal evaluation of the CIE procedure, and may prove useful in difficult cases where extreme sensitivity is required, as with post-treated patients returning for testing with recurring symptoms of their originally diagnosed disease. Bacteriology labs have difficulty in growing the disease organism of post-treated patients and CIE becomes an important diagnostic method requiring the greatest sensitivity the test can give.

EXPERIMENTAL

ELECTROPHORESIS BUFFER SOLUTION:

Diethyl barbituric acid and sodium barbitol (pH 8.6, ionic strength 0.05). Prepackaged, purchased from Fisher Chemical Co., St. Louis, Missouri, package brought to 1000.0ml. with deionized water. Stored at 4.0°C. until used.

AGAROSE:

Dissolved 1.0g. agarose, Fisher Chemical Co., St. Louis, Missouri, in 100.0ml. buffer solution, boiled on hot plate until all agarose dissolved. Electrophoresis grade agarose.

PREPARATION OF ELECTROPHORESIS PLATE:

Glass plates, 10.2cm. x 8.3cm. x 1.5mm., were covered with 13.5ml. of the hot melted agarose. Average depth of the agarose upon cooling 1.5mm. Wells were cut into the agarose with a hole punch, (GraFar Corp., Detroit, Michigan). Wells were 3.0mm. in diameter and 1.0cm. apart as measured from the center of each well. The plates were stored in a humidity chamber, (GraFar Corp.), at 4.0°C. until used.

ANTISERA:

Hemophilus influenzae type b diluted 1/30 in sterile physiological saline. Antisera supplied by Dr. Shehla Naqvi of Cardinal Glennon Hospital for Children, St. Louis, Missouri. Stored at 4.0°C. until used.

CLINICAL SPECIMENS:

CSF specimens were collected from patients admitted to Cardinal Glennon Hospital for Children, St. Louis, Missouri. All specimens were stored at -20.0°C. until used. All specimens were known to be positive for H. flu type b.

COUNTER-CURRENT IMMUNOELECTROPHORESIS:

Each specimen was thawed at room temperature and serial dilutions were made using sterile physiological saline. 10.0ul. of the serum or CSF was placed into the cathode side wells of the electrophoresis plate and the antisera, 10.0ul., in the anode side wells. Electrophoresis was carried out using a Gelman Electrophoresis Chamber and Power Supply product number 51211, (Gelman Instruments Co., Ann Arbor, Michigan). The agarose plates were connected to the electrophoresis chamber buffer tank by wicks made from Whatman No. 3 filter paper. A constant amperage of 12.0mA. for 45.0 minutes was applied across the plate. All electrophoresis was carried out at room temperature. After electrophoresis, the plates were viewed visually using a darkground viewer, (Hyland Viewer, Travenol Labs, Costa Mesa, California). Control plates were soaked in sterile physiological saline overnight at 4.0°C. before final viewing.

ULTRAVIOLET PHOTOGRAPHY:

The agarose gel was removed from the glass plate and placed upon a piece of cellophane (Dow Co., Indianapolis, Indiana). By sliding a razor blade between the agarose and the glass, it was possible to remove the gel in one piece. The agarose with the cellophane underneath was placed upon a 4x5 inch sheet of Kodak Commercial 6127 film, (Eastman Kodak Co., Rochester, New York), and exposed with an ultraviolet lamp, (Ultraviolet Products Inc., Mineralight, San Gabrial, California), at a distance of 115.0cm. for one second. A sheet of cardboard was used to control the exposure. The film was taped to the bottom of a plastic tray; developed in Kodak D-11 at 20.0°C. for 8.0 minutes with constant tray rock agitation; fixed using Kodak F-6 for 4.0 minutes with intermittent agitation; and soaked in a solution of

Perma Wash, (Heico Inc., Delaware Water Gap, Pennsylvania). The film was then washed with tap water for 30 seconds, soaked in Kodak Photo-flo for 30 seconds, and clipped on a hanger to air dry. The film was examined under the darkground viewer used to read the agarose plates.

AMIDO BLACK STAIN:

After electrophoresis, the gel was washed for 3 hours in saline, 0.9% NaCl, dried at 37°C., and then soaked in 0.5% amido black 10B stain, (Sigma Chemical Company, St. Louis, Missouri), made in 10% acetic acid for 5 minutes. The excess amido black was then removed from the gel matrix by rinsing in 5% acetic acid and deionized water. Afterward, the gel was stored at 4.0°C in the humidity chamber until used.

ACID BUFFER WASH:

Prior to tannic acid treatment, the gel was washed for 10 minutes in an acid buffer. The gel was placed in a 4 liter glass jar with 1 liter of the acid buffer and stirred with a magnetic stir bar for 10 minutes. By keeping the speed of the stir bar slow the gel was undamaged by this process. Electrodes of a Beckman Zeromatic pH meter, (Beckman Instruments, Fullerton, California), were taped to the jar and constant pH measurements were taken. The acid wash was made from McIvaine's standard buffer solutions.[13] By varying the amounts of a 0.1 molar citric acid solution and a 0.2 molar disodium phosphate solution, buffer solutions from 2.2 to 8.0 pH can be made, (Table 1).

TANNIC ACID TREATMENT:

Dissolved 0.25g. tannic acid, (Sigma Chemical Co., St. Louis, Missouri), in 100ml. deionized water. Agarose gel was soaked for 15 minutes in the tannic acid solution.

RESULTS

Testing showed that direct-contact ultraviolet photography of the agarose gel upon which CIE was performed produced a decrease in the sensitivity of the test by two serial dilutions when compared to the control plate. Staining the precipitate formed by the antigen/antisera reaction with Amido 10B increased the film sensitivity by one additional serial dilution when compared to the film of the unstained gel, but still one dilution less than could be visually observed on the gel itself.

By treating the gel in a 0.25% tannic acid solution, it was possible to detect on the film the last precipitate line that could be seen visually on the gel, but showed no increase in the overall sensitivity. It was found that the tannic acid treatment did increase the sensitivity of the visually observed results on the gel itself. A white opaque band was formed around the antisera well and obscured the precipitate line formed by the antigen/antisera reaction. Repeated use of the tannic acid solution caused the solution to turn a greenish-brown color, and a decrease of sensitivity of the visually observed gel was noted. Adjusting the pH of the tannic acid solution above pH 8.0 with sodium hydroxide gave the same result as repeated use of the tannic acid. The obscuring precipitate in the gel was found to be removed by soaking the agarose gel with constant agitation for ten minutes in a pH 4.0 McIvaine's buffer solution. No color change of the tannic acid was observed after repeated use. The McIvaine's buffer solution remained at pH 4.0 after repeated use.

Seven patients' specimens of CSF were twice tested using the acid buffer wash and the tannic acid treatment. Of the 14 tests run, 4 showed an increase in sensitivity of two serial dilutions, 8 showed an increase of

one serial dilution, and 2 showed no increase in the sensitivity over that of the control, (Table II). Different types of antisera required acidic buffer washes at various pH's. Meningococcal Groups A, C, D, (Burroughs Wellcome Labs, Bechenham, England), needed an acid buffer wash at pH 3.0 to remove the obscuring precipitate-causing antisera. Pneumococcal OMNI serum, (Statens Seruminstitut, Copenhagen, Denmark), required an acid buffer wash at pH 2.6.

The enhancement of the precipitate line with tannic acid treatment reached a maximum after 10-15 minutes. Longer treatment times, up to 8 hours, gave no greater enhancement. Soaking the agarose gel in physiological saline at 4^{o}C. overnight reduced the precipitate formed around the antisera well, but did not remove it completely.

DISCUSSION

Direct-contact ultraviolet photography is not a useful method to increase the sensitivity of counter-current immunoelectrophoresis. Greater amounts of protein than present in the line formed by the antigen/antisera reaction are needed to form a noticable density difference on film caused by the absorption of ultraviolet radiation by the proteins. With tannic acid treatment of the gel it was possible to detect on film the faintest line that could be visually discerned and may be useful as a system for the permanent recording of the test. Tannic acid has been shown [14-17] to improve the detection of precipitin protein lines as formed in electrophoretic gels. For the problem of background opacity produced by the reaction of the tannic acid and the antisera [18-19] it was suggested that the gel first be washed in a saline solution for several hours to overnight prior to the tannic acid treatment. The overnight soak in saline proved not to be completely effective in removing the antisera causing precipitate and greatly increased the time for completion of the test. With a 10 minute wash in a acidic buffer solution no precipitate was formed around the antisera well and it appears to represent a new method for using tannic acid to improve the detection of precipitin lines formed by antisera/antigen reactions. Increasing the pH of the tannic acid reduces the effectiveness of the method and actually reduces the CIE overall sensitivity. The electrophoretic buffer in the agarose could raise the pH of the tannic acid if not first removed from the gel matrix. The acid buffer wash lowers the pH of the gel reducing the possibility of this problem. The mechanism by which the acid buffer wash removes

the antisera forming precititate is not clear, but, probably results from an increase in the solubility of the antisera by a lowering of the pH. Each antisera appears to have a distinct pH range that increases its solubility enough to remove it from the gel matrix. This causes practical problems for labs where several different antisera are used at once on the same gel. It may be possible to use one particular pH acid buffer wash to remove all the antisera forming precipitate but further research needs to be done to determine this.

Using a scale of 0 for no increase in sensitivity, 1 for a increase of one dilution in sensitivity, and 2 for a increase of two dilutions in sensitivity, the average, $\bar{x}$, increase in sensitivity was 1.1 dilutions. The standard deviation, s, was 0.66. Using a table of Student's t distribution[20] it can be stated that the probability of a increase in sensitivity, using tannic acid treatment with acid buffer wash, is between 0.7 dilutions and 1.4 dilutions with a 95% confidence level.

REFERENCES

1. D. J. Gocke, and C. J. Howe, "Rapid Detection of Australia Antigen by Counterimmunoelectrophoresis", J. Immunol., 104: 1031-1032, 1970.

2. E. A. Edwards, P. M. Muehl, and R. O. Peckinpaugh, "Diagnosis of Bacterial Meningitis by Counterimmunoelectrophoresis", J. Lab. Clin. Med., 80: 449-454, 1972.

3. B. M. Greenwood, H. C. Whittle, and O. Dominic-Rajkovic, "Counter-Current Immunoelectrophoresis in the Diagnosis of Meningococcal Infections", Lancet, 519-521, 1971.

4. G. J. Dorff, J. D. Coonrod, and M. W. Rytel, "Detection by Immuno-electrophoresis of Antigen in Sera of Patients with Pneumococcal Bacteremia", Lancet, 578-579, 1971.

5. J. D. Coonrod, and M. W. Rytel, "Specificity of Counter-Immunoelectrophoresis in Bacterial Meningitis", Lancet, 829, 1972.

6. D. L. Ingram, P. Anderson, and D. H. Smith, "Countercurrent Immunoelectrophoresis in the Diagnosis of Systemic Diseases Caused by Hemophilus Influenzae Type b", J. Peds., 81: 1156-1159, 1972.

7. K. Shimao, "The Estimation of Serum Proteins by Ultraviolet Densitometry of Agar Electrophoretic Diagram", J. Biochem., 49: 451-455, 1961.

8. N. Ressler, "Photoelectric Ultraviolet Photometry Applied to Protein Electrophoresis", J. Lab. & Clin. Med., 54: 291-299, 1959.

9. N. Ressler, and S. D. Jacobson, "Far Ultraviolet Direct Photometry Applied to Protein Electrophoresis", J. Lab. & Clin. Med., 54: 1959.

10. S. J. Luner, "Ultraviolet Photography for Visualization of Separation Patterns of Zone Electrophoresis and Other Methods", Anal. Biochem., 23: 357-358, 1968.

11. S. M. Hassur, and H. W. Whitlock, "Direct-Contact Photography--A Convenient and Versatile Technique for the Cameraless Photography of Polyacrylamide Gels", Anal. Biochem. 62: 609-614, 1974.

12. W. J. MacLennon, G. P. Miller, and S. Lewis, "Quantitating Electrophoretic Protein Using Photographic Records", Med. Biol. Illus. 19: 185, 1969.

13. C. D. Hodgman (Editor In Chief), Handbook of Chemistry and Physics, Chemical Rubber Publishing Co., Cleveland, Ohio, 1943 p. 1336.

14. R. Hopkins, and P. C. Das, "Improved Sensitivity of the Electrophoresis Method by Tannic Acid for Detection of Australia Antigen", J. Clin. Pathol., 25: 832-833, 1972.

15. K. Aoki, S. Kajiwara, R. Shinke, and H. Nishira, "Tannic Acid Staining and Extraction of Enzymes in Polyacrylamide Gel Electrophoresis", Anal. Biochem., 95: 575-578, 1979.

16. P. J. Middleton, M. Petric, C. M. Hewitt, M. T. Szymanski, and J. S. Tam, "Counter-immunoelectro-osmophoresis for the Detection of Infantile Gastroenteritis Virus (Orbi-Group) Antigen and Antibody", J. Clin. Path., 29: 191-197, 1976.

17. E. Alpert, M. Monroe, and P. H. Schur, "A Method of Increasing the Sensitivity of Radial-Immundiffusion Assay", Lancet, 1120, 1970.

18. Ibid. #16

19. Ibid. #17

20. A. D. Rickmers, and H. N. Todd, Statistics An Introduction, McGraw-Hill Book Co., New York, 1967, p. 553.

APPENDIX

TABLE I

McIVAINE'S STANDARD BUFFER SOLUTIONS

Stock solution A: 0.1 molar citric acid solution.
Stock solution B: 0.2 molar disodium phosphate solution.

pH	Soln. A cc	Soln. B cc	pH	Soln. A cc	Soln. B cc
2.2	19.60	0.40	5.2	9.28	10.72
2.4	18.76	1.24	5.4	8.85	11.15
2.6	17.82	2.18	5.6	8.40	11.60
2.8	16.83	3.17	5.8	7.91	12.09
3.0	15.89	4.11	6.0	7.37	12.63
3.2	15.06	4.94	6.2	6.78	13.22
3.4	14.30	5.70	6.4	6.15	13.85
3.6	13.56	6.44	6.6	5.45	14.55
3.8	12.90	7.10	6.8	4.55	15.45
4.0	12.29	7.71	7.0	3.53	16.47
4.2	11.72	8.28	7.2	2.61	17.39
4.4	11.18	8.82	7.4	1.83	18.17
4.6	10.65	9.35	7.6	1.27	18.73
4.8	10.14	9.86	7.8	0.85	19.15
5.0	9.70	10.30	8.0	0.55	19.45

TABLE II

RESULTS AS REPORTED IN DILUTIONS

Sample	Control	Tannic Acid Treatment
#1 CSF	1/16	1/64
	1/8	1/32
#2 CSF	1/128	1/256
	1/256	1/512
#3 CSF	1/128	1/256
	1/128	1/256
#4 CSF	1/64	1/128
	1/128	1/256
#5 CSF	1/256	1/1024
	1/256	1/1024
#6 CSF	1/64	1/128
	1/64	1/128
#7 CSF	1/256	1/256
	1/256	1/256

VITA

James Edward Hohenberger from St. Louis, Missouri received the B.A. degree in Biology from St. Louis University. He was employed at Cardinal Glennon Hospital for Children in St. Louis, Missouri with responsibilities of testing for meningococcal infections in the Infectious Disease Lab using counter-current immunoelectrophoresis. At the same time, he worked as a laboratory technician for the Research Hematology Lab and co-authored a publication in the American Journal of Clinical Pathology, Vol. 74, No. 4, 1980. He is currently working toward a Bachelor of Science degree in the Photographic Science and Instrumentation program at Rochester Institute of Technology in Rochester, New York.