Immunoperoxidase Staining of Leptospires

D. N. TRIPATHY AND L. E. HANSON
College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801

Leptospires were detectable by light microscopy when Formalin-fixed preparations were reacted by direct and indirect methods with horseradish peroxidase-labeled specific antibody.

Silver impregnation staining is one of the principal methods used for staining of leptospires, since these organisms are difficult to stain with the usual bacterial stains. However, this method is not very specific, since certain artifacts may be mistaken for leptospires. Fluorescent antibody staining has been used to detect leptospires in urine and in tissue specimens (1). Another antigen-antibody reaction which has been used in detection of viral antigens is the enzyme labeling technique (4, 5). This method has the advantage that the antigen and antibody reaction, unlike the fluorescent antibody method, can be examined by light microscopy. The present report describes preliminary investigations on use of the immunoperoxidase technique for detection of leptospires.

Specific antiserum against serotypes *grippotyphosa* and *andamana* produced in rabbits was labeled by the methods of Wicker and Avrameas (5). The serum was fractionated with saturated ammonium sulfate three times at pH 7.8 and finally dissolved in borate-buffered saline to half the original volume. It was then dialyzed in borate-buffered saline in the cold until it was shown to be free of sulfate ions by reacting the dialysate with 10% barium chloride. Rabbit anti-bovine immunoglobulin G was obtained from Miles Laboratories Inc. and was labeled with horseradish peroxidase.

For conjugation of antibody, 10 mg of horseradish peroxidase (Miles Seravac Laboratories [Pty.] Ltd.) was added to 1 ml of 0.1 M phosphate buffer, pH 6.8, which contained 5 mg of antibody preparation. To this was added 0.05 ml of a 1% aqueous solution of glutaraldehyde (2) drop-wise, and the preparation was held at room temperature for 2 h. It was then dialyzed overnight against two changes of phosphate-buffered saline, pH 7.2.

Cultures of leptospires grown in bovine albumin polysorbate 80 (3) medium for a week at 29 C were fixed in Formalin-saline solution, and smears were prepared on clean slides. The slides were held at room temperature for at least 15 to 18 h.

For the direct staining method, a 1:10 dilution of peroxidase antibody preparation in normal saline was added to the smear, which was then incubated for 30 to 50 min in a moist chamber at 37 C. The slides were washed two times for 5 to 10 min, air-dried, and then reacted with benzidine reagent (4) (consisting of 1 ml of 5% ethylenediaminetetraacetic acid, 1 ml of saturated ammonium chloride, 9 ml of saturated benzidine solution, and 1 drop of 3% hydrogen peroxide) for 4 to 5 min. The smears were washed and mounted in buffered glycerine. For the indirect method, the smears of *grippotyphosa* were first reacted for 30 to 50 min at 37 C with bovine antiserum against serotype *grippotyphosa* having a microscopic agglutination titer of 1:1,000. They were then washed two times with distilled water and reacted with peroxidase-labeled rabbit anti-bovine immunoglobulin G. After washing, the smears were reacted with benzidine reagent and mounted. The stained smears were examined with a light microscope. The mounted, labeled preparations were stored in the refrigerator for future observation.

Leptospires were detected in the smears by both the direct and indirect immunoperoxidase technique.

![Fig. 1. Leptospira grippotyphosa stained by an indirect immunoperoxidase technique.](image-url)
methods. The organisms stained yellowish-brown to dark brown. Leptospires stained more intensely by the indirect technique (Fig. 1) than by the direct method.

Leptospires were detectable in immunoperoxidase-stained preparations that had been stored in a moist environment in the refrigerator for as long as 4 months. The principal advantage of this method is that the preparation can be visualized by using a light microscope, whereas ultraviolet light is needed for the immunofluorescent staining method. Further, stained preparations can be kept for a longer period of time without loss of staining. Studies on the use of this method on infected tissues are in progress.

This investigation was supported by funds from the Illinois Agricultural Experiment Station Hatch project 30-15-70-302.

LITERATURE CITED
1. Alexander, A. D., W. S. Gochenour, K. R. Reinhard, M. K. Ward, and R. H. Yager, 1970. Leptospirosis, p. 382–421. In H. L. Bodily, E. L. Updyke, and J. O. Mason (ed.), Diagnostic procedures for bacterial, mycotic and parasitic infections, 5th ed. American Public Health Association, Inc., New York.
2. Avrameas, S. 1969. Coupling of enzymes to proteins with gluteraldehyde. Use of the conjugate for detection of antigen and antibodies. Immunochemistry 6:43–52.
3. Ellinghausen, H. C., Jr., and W. G. McCullough. 1965. Nutrition of Leptospira pomona and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Amer. J. Vet. Res. 26:45–51.
4. Kurstak, E. 1971. The immunoperoxidase technique: localization of viral antigens in cells, p. 423–444. In K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press Inc., New York.
5. Wicker, R., and S. Avrameas. 1969. Localization of virus antigens by enzyme-labelled antibodies. J. Gen. Virol. 4:465–471.