APPLIED MICROBIOLOGY, Mar. 1973, p. 427-430
Vol. 25, No. 3
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Procedure for Drying Leptospiral Antibody on Sand and Sugar for Serological Studies in Leptospirosis

DONALD M. MYERS

Pan American Zoonosis Center, Pan American Health Organization, Ramos Mejia, Buenos Aires, Argentina

Received for publication 28 December 1972

A simple technique is described for drying sera on washed, dry sand or ordinary sugar cubes for the sero-diagnosis of leptospirosis. The results are shown to be very similar to those obtained with fluid sera or sera dried on filter paper discs. Sera adsorbed on sand or absorbed in sugar eliminated some of the problems associated with sera dried on paper. This method is suitable for use in the field and is expected to be of value in epizootiological studies where contamination and chemical denaturation of fluid serum samples held without refrigeration is a problem.

Filter paper has been used as a technique for drying antibody for several viral diseases (1, 4, 5, 7). The method was reported to be of value in leptospiral serological survey studies (6) and eliminated problems involved in handling and shipping fluid serum samples. One of the disadvantages that has been associated with the paper disc technique is the need for time-consuming methods to elute all antibody from the paper. In addition, the pH of the eluting fluid and the chemical composition of the paper used may adversely affect titers.

The purpose of this paper is to report the successful application of sand and sugar to the collection, drying, and testing of sera for leptospiral antibody. Sand was selected for study because of its adsorbing and inert quality when dry and its ability to allow complete elution of dried serum samples. Sugar was included because of its bacteriostatic action and its solubility without adversely affecting antibody titer. Results obtained with these two substrates compared favorably with results obtained using the filter paper disc method. Drying sera on either sand or sugar was found to be as effective in preserving antibody as drying on filter paper and eliminated some of the problems associated with the latter.

MATERIALS AND METHODS

The *Leptospira* serotype *hardjo* strain Hardjoprajitino from our reference culture collection was used throughout this study. This strain was used as antigen because a high percentage of bovine sera possesses antibody too.

Sera. Bovine sera obtained from leptospirosis surveys were used to compare the effectiveness of sand, sugar, and filter paper discs for preserving leptospiral antibodies. The sera were previously examined by the microscope agglutination test and on the basis of titers to *hardjo*. Ten pools were prepared, each consisting of ten bovine sera with like titers ranging from 1:100 to 1:1,600 in each pool. One pool of 10 sera that did not show leptospiral antibody was used as a negative control, and one hyperimmune rabbit serum prepared by intravenous injection of *hardjo* cells was also used. The pooled sera were adsorbed onto sand, sugar, and filter paper disc, and then stored at −30 C. Thawed fluid sera were retested for reproducibility of titers after 1, 4, and 8 weeks. Prior to testing, samples were thawed and diluted with phosphate-buffered saline (pH 7.2) to equal a 1:25 serum dilution. Serological examination of both fluid sera and eluates was done by the microscope agglutination test by using live *hardjo* antigen according to the procedure used by Galton et al. (3); serial twofold dilutions were made in the buffered saline to determine antibody titers.

Preparation of dried samples: sand. Common beach sand was washed repeatedly in running tap water until the water was free of particulate matter by dark field examination. The washed sand was then rinsed with distilled water, dried, and approximately 1.5 g was dispensed into 1-dram screw-cap vials. In some tests the washed sand was treated before drying with a 0.5% solution of phenol in distilled water to study the effect of the preservative on leptospiral antibody.

Nine vials containing 0.25 ml of each serum pool including the rabbit antiserum were prepared. The sera were allowed to adsorb into the dry sand, dried without caps overnight in an incubator (37 C) and then the vials were sealed. Triplicates of each of the dried samples were stored at 5 C, room temperature,

and at 37 C and tested at 1, 4, and 8 weeks to compare the effect of time and temperature on titers during storage. Samples were eluted by adding buffered saline (pH 7.2), shaking for 30 s and then transferring the mixtures to test tubes and adjusting the volume of the eluate to 6.0 ml. The sand rapidly settled to the bottom of the tube, leaving the supernatant fluid which was considered equivalent to a 1:25 dilution of serum. In addition, the contents of some vials containing sera adsorbed onto sand were transferred directly to test tubes before the addition of the eluting fluid to evaluate the possibility of a drop in titer caused by antibody adhering to the glass.

Sugar. Test sera in a volume of 0.25 ml were pipetted onto ordinary table sugar cubes which averaged 3.0 g each. The cubes were placed in open plastic bags, dried by overnight incubation (37 C) and then the bags were sealed and stored in the same way as the sand and the fluid samples. Elution was accomplished by transferring the cube to a small screw-cap bottle containing sufficient buffered saline to give a final volume of 6.0 ml. The sugar cubes were allowed to completely dissolve at room temperature to give approximately a 1:25 serum dilution.

Filter paper discs. Filter paper discs (Whatman 2) having a diameter of 32 cm were used for drying the serum pools. Saturation was accomplished by dropping 0.25-ml volumes from a pipette onto marked areas of the filter paper. The saturated filter papers were dried overnight at room temperature. Discs were then cut to include the entire area of diffused sera, sealed in plastic bags, and stored as the sand and sugar samples. Dried paper disc samples were placed in test tubes containing 6.0 ml of the buffered saline, shaken, and then refrigerated at 5 C overnight to allow the antibody to elute. The following day the test tubes were again shaken and the filter paper discs were removed with forceps.

RESULTS

The *hardjo* leptospiral antibody was found to be quite stable when sera are dried and stored on sand, sugar, or filter paper discs. The serum pools ranging in titer from 1:100 to 1:1600 which had been adsorbed onto sand or sugar showed titers equal to or differing by no more than one serum dilution from those obtained with fluid sera after storage for 7 days at 5 C, room temperature, and at 37 C. Two of the serum pools dried on filter paper showed a fourfold drop in titer when stored at 37 C, but not when stored at 5 C or at room temperature. After 8 weeks of storage, no significant loss in activity was evident in either the sand-adsorbed or sugar-absorbed samples regardless of the temperature at which they were maintained. More variation was observed with the sera dried and stored on paper discs when titers were compared with those of fluid sera. After 8 weeks, two of the paper-dried serum pools demonstrated a fourfold drop in titer after storage at room temperature, and six samples, including the hyperimmune serum, showed a fourfold loss in titer at 37 C.

The results obtained with replicate tests on rabbit hyperimmune *hardjo* serum dried and stored for 8 weeks on sand, sugar, and filter paper discs are summarized in Table 1. The lower titers obtained with the paper disc method appear to reflect incomplete elution rather than instability of antibody upon drying since the titer obtained with sand and sugar eluates were identical to those of the fluid serum.

Statistical data were analyzed without considering temperatures and storage periods, since these factors apparently do not affect the elution of the serum antibodies. Titers obtained with sera dried on filter paper and on sand were compared with agglutination titers of the frozen control sera (Tables 2 and 3). To facilitate computations, the dilutions were modified dividing by 50 the $\log_2$ of the reciprocals. The zero value was conventionally assigned to sera negative at 1:100 dilution. Against this modified scale, regression lines were adjusted. Assuming that the method under study will indicate zero in the absence of antibodies, the adjustment was made with the condition that the lines must pass through the origin (point 0.0). In the case of perfect agreement, that is if titers of the control sera correspond to titers of test sera, the slope of the line is 1. Results of titrations of sera dried on sand showed a line (Ys) with a slope of 0.99 indicating a very high degree of coincidence (Fig. 1). For sera dried on filter paper, the slope of the line (Yp) was 0.80, which shows a reduced value with respect to the titers of the control sera. The statistical test comparing the two slopes showed a highly significant difference ($P < 0.01$).

Results obtained with sugar cubes were very similar to those obtained with sand.

One of the advantages of paper discs is that they are fast drying and tend to control contaminants. The dried discs can be mailed in an envelope to a laboratory for testing. Contamination of sand- or sugar-dried samples did not present problems in the performance of the test when drying was under controlled overnight incubation. Eluates from dry sand previously treated with a phenol-water rinse showed no evidence of contamination or loss in antibody activity even when drying was conducted at room temperature. Sand-dried samples which were eluted in the storage vials had titers comparable to those of samples which were

TABLE 1. *Results of titers obtained with leptospiral rabbit hyperimmune hardjo antiserum[a] dried on sand, sugar and filter paper after 8 weeks of storage at varying temperatures*

Method of drying	Storage temperature		
	Refrigerator 5 C	Ambient (approx 25 C)	Incubator (37 C)
Sand	25,600[b]	25,600	25,600
Sugar	25,600	25,600	25,600
Filter paper disc	12,800	12,800	6,400

[a] Fluid serum titer, 1:25,600.
[b] Reciprocal of titers.

transferred to test tubes prior to elution, suggesting that an insignificant amount of antibody adheres to the glass vial. For convenience in mailing, adsorption and elution may be carried out in plastic bags, thus eliminating any objection to the use of glass vials.

DISCUSSION

The data presented show that the use of sand or sugar for drying serum samples gives results very similar to those obtained with fluid serum or with the filter paper disc method. Although the paper disc has been shown to be a convenient method for handling dried serum, several disadvantages have been associated with it which may cause a drop in titers. Karstad et al. (4) have shown that varying the elution time from 30 min to overnight affected resultant titers. The same authors also found that by compression of the discs with the tip of a pipette, viral neutralization titers of eluants were consistently raised. The hemagglutination and complement-fixation tests used by Chin et al. (2) with paper-dried sera involved both

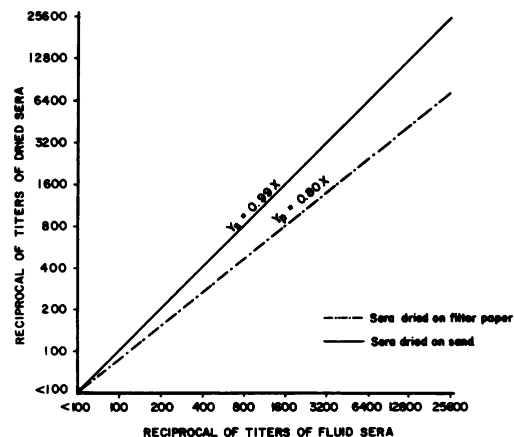

FIG. 1. *Regression lines of titers of sera dried on paper filter and sand on the titers of control sera.*

TABLE 2. *Correlation between titers of sera dried on filter paper and control sera*

Titers of the pool of control sera	Titers of sera dried on filter paper									
	<100	100	200	400	800	1,600	3,200	6,400	12,800	25,600
<100	9									
100	9	0								
200	1	4	4							
400	1	4	11	11						
800			4	17	6					
1,600				3	14	1				
25,600[a]								3	4	2

[a] Rabbit hyperimmune serum.

TABLE 3. *Correlation between titers of sera dried on sand and control sera*

Titers of the pool of control sera	Titers of sera dried on sand									
	<100	100	200	400	800	1,600	3,200	6,400	12,800	25,600
<100	9									
100		9								
200			9							
400			5	19	3					
800					27					
1600					4	14				
25,600[a]										9

[a] Rabbit hyperimmune serum.

overnight elution and compression of the disc in a plastic syringe to achieve maximal antibody titers. Kingscote (6), in assessing the paper disc technique for leptospirosis surveys, found the pH of the buffer used for elution affected titers. When antibody was eluted with a buffer of pH 7.0, the titers were equal to those of corresponding fluid serum. However, when the discs were eluted with a pH 7.6 buffer, eluates were found to be threefold dilution lower in titer. A variety of types of both filter or blotting paper has been used for the disc method, and little is mentioned concerning the possibility of chemical contamination or pH changes resulting from the manufacturing process, both of which may adversely affect antibody titers. In addition, the work involved at a receiving laboratory in preparing eluates from paper discs must be considered, especially when a large number of samples is involved.

Sand or sugar is easier to work with than filter paper discs. Methods employing these substrates are not particularly time-consuming; antibody elutes rapidly and titers of eluates compare with those of original fluid sera. These procedures appear to offer an improved, convenient, and uniform method for the collection, handling, and shipping of dried serum for leptospiral antibody surveys, eliminating many of the problems which can be expected with fluid serum samples held without refrigeration.

ACKNOWLEDGMENTS

I am indebted to Naum Marchevsky of the Pan American Zoonosis Center for the statistical analysis of the data and to Rexford D. Lord for his stimulating discussions.

LITERATURE CITED

1. Adams, E., and R. P. Hanson. 1956. A procedure for adsorbing virus neutralizing antibodies on paper discs. J. Bacteriol. 72:572.
2. Chin, J., N. H. Schmidt, E. H. Lennette, and M. Hanahoes. 1966. Filter paper disc method of collecting whole blood for serologic studies in children. Amer. J. Epidemiol. 84:74-80.
3. Galton, M. M., R. W. Menges, E. B. Shotts, A. H. Nahmias, and C. W. Heath. 1962. Leptospirosis: epidemiology, clinical manifestations in man and animals and methods in laboratory diagnosis. U.S. Public Health Service Publ. No. 951, Washington, D.C.
4. Karstad, L., J. Spalatin, and R. P. Hanson. 1957. Application of the paper disc technique to the collection of whole blood and serum samples in studies on eastern equine encephalomyelitis. J. Infect. Dis. 101:295-299.
5. Karstad, L., S. Vadlamudi, R. P. Hanson, D. O. Trainer, Jr., and V. H. Lee. 1960. Eastern equine encephalitis studies in Wisconsin. J. Infect. Dis. 106:53-59.
6. Kingscote, B. F. 1969. Leptospirosis survey in a white-tailed deer herd in Ontario: comparative use of fluid and paper disc-absorbed blood. Bull. Wildlife Dis. Ass. 5:68-72.
7. Worth, R. M. 1964. The filter disc blood collection method adapted for a mumps HAI serologic survey. Amer. J. Hyg. 79:245-249.