Public Health Reports

Vol. 66 • SEPTEMBER 21, 1951 • *No. 38*

Preparation of Dried Antigen and Antiserum for the Agglutination-Inhibition Test for Virus Influenza

By M. R. HILLEMAN, Ph.D., E. L. BUESCHER, Captain, M. C. and J. E. SMADEL, M. D.*

Army Area and Medical General Laboratories of the United States Army were supplied with influenza diagnostic antigens in liquid form between 1946 and 1948. Such materials were not completely satisfactory because their keeping qualities were somewhat variable and unpredictable. The stability of influenza virus hemagglutinin on desiccation (*1*) has been utilized in the preparation of a satisfactory dried product which has been used in the Army laboratories since 1948. Detailed information on the preparation and stability of these antigens and their control antisera is presented here.

Preparation of Antigens

The standard type A (PR8), A prime (FM1), and type B (Lee) strains of influenza virus (*2*) supplied by the Influenza Strain Study Center [1] of the Armed Forces Epidemiological Board are used as seed material. Detailed information on the characteristics of these viruses is given in the Catalogue of the Viral and Rickettsial Registry of the American Type Culture Collection (*3*). The seed virus preparations, which are not more than 10 passages removed from propagation in the mouse, are maintained as infected allantoic fluid either stored at −70° C. in sealed glass ampules or dried.

Nine-day embryonated eggs from chickens free of *Salmonella pullorum* infection are inoculated in groups of 50 into their allantoic cavities with 0.4 cc. amounts of a dilution of the appropriate seed virus (usually 10^{-4} to 10^{-5}) which has been found in preliminary titrations to produce chorioallantoic fluids with hemagglutinin titers (*2*)

---

*From the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington, D. C., with the technical assistance of W. O. Blair, S.F.C., A.M.S., and J. R. Hitaffer.

[1] Located at the laboratory of Dr. T. P. Magill, State University of New York Medical Center, 335 Henry Street, Brooklyn 2, N. Y.

NOTE: The trade names appearing in this study are carried as a means of identifying the products under discussion and do not represent endorsement of the product by the Public Health Service.

of 1:320 or greater. After further incubation at 35° C. for 44 to 48 hours, the eggs are candled and the embryos which are still alive are chilled overnight in the refrigerator.

The allantoic fluids, harvested from the eggs remaining from each initial group of 50, are pooled and then heated at 37° C. for 1 hour to assist elution of virus from the small amounts of red blood cells and other particulate matter inadvertently included in the fluids during harvest. They are then centrifuged at 1,000 rpm for 10 minutes in the horizontal machine. Duplicate tubes of standard National Institutes of Health fluid thioglycollate medium (*4*) are inoculated with 1.0 cc. amounts of centrifuged allantoic fluid and then sufficient 1:20 Merthiolate (Lilly) solution is added to the pools to give a final concentration of 1:10,000.

After 5 days storage at 4° C. to await the outcome of the cultural tests, the sterile fluids are selected and warmed to 37° C. in order to redissolve precipitated salts. These are pooled to form the final batch and distributed in 5.0 cc. amounts into sterile Pyrex glass ampules of approximately 10.0 cc. capacity (with inside neck diameter not less than 8 mm.) or into 14 cc. glass bottles (S27AB, T. C. Wheaton Co., Millville, N. J.). After shell freezing in the alcohol-dry ice bath, the ampules or bottles are placed on or in the freeze-drying machine. When the drying process cannot be started at once, the distributed material may be stored at either —20° C. or —70° C. for a period of not longer than 5 days.

Considerable experience has now shown that with the procedure outlined above, the over-all average yield of satisfactory sterile infected allantoic fluid is about 5.0 cc. per harvested egg. It may be necessary to discard as much as 10 percent of the fluid obtained because of positive bacterial cultures and from 1 to 10 percent of the eggs are lost because of death prior to harvest of fluid. The lowest incidences of premature deaths and bacterial contamination are generally obtained during the winter and early spring and the highest incidences during the late summer and early fall.

Preparation of Rooster Antiserum

Pairs of 6–8 lb. roosters are injected both intravenously and intraperitoneally with 5.0 cc. amounts of the appropriate freshly harvested allantoic fluids having hemagglutinating titers of 1:320 or greater. Ten days later, the roosters are bled by cardiac puncture using a 100-cc. syringe or a vacuum bottle with proper connections. Yields of blood of from 80 to 125 cc. are obtained. The clotted blood is cut into pieces approximately 1 cm. in diameter and stored at 4° C. overnight. On the following day, the clotted blood is allowed to stand at room temperature for 4 or 5 hours to promote shrinkage of the clot

and is then centrifuged, and the serum harvested. Usually the volume of the serum is one-fourth to one-third that of the whole blood.

To be satisfactory, antisera must have a titer of 1:800 or more when tested with homologous antigen in the agglutination-inhibition test (see below). Sera with titers of 1:3200 or greater are usually diluted with normal rooster serum to give final titers of 1:800 or 1:1600. The satisfactory sera are inactivated at 56° C. for 35 minutes, dispensed in 0.5 cc. amounts into 5.0 to 10.0 cc. Pyrex glass ampules, plug frozen, and are either immediately placed on the drying machine or stored at −20° C. or −70° C. until dried (within 2 weeks). Approximately 50 to 60 percent of the roosters injected give antisera of satisfactory potency.

It is important to mention in this connection that the roosters used for preparing antisera must be fed a diet containing sufficient amounts of calcium to insure adequate contraction of the blood clot. A satisfactory diet used in this laboratory consists of equal amounts of scratch feed and growing mash and is supplemented by crushed oyster shell and sea sand.

Drying Procedure

Two kinds of apparatus have been used for drying the influenza antigens and antisera. One is a modification of the unit originally described by Bauer and Pickels (*5*) and is intended for use with ampules to be sealed with a torch. This manifold drier was employed in the department for the large-scale drying of Japanese encephalitis vaccine (*6*). The other is a Stokes machine of the chamber type (*7*) designed for drying materials in bottles to be sealed with rubber closures. All influenza diagnostic materials distributed to Army laboratories since 1948 have been dried in the apparatus of Bauer and Pickels; the Stokes equipment has been used only to dry experimental lots of antigen.

Desiccation with the manifold drier is carried out as follows:

The ampules containing frozen antigen or antiserum are chilled with crushed dry ice for an hour and then rapidly loaded on the manifolds which are placed in the refrigerated compartments that have been precooled to about −20° C. The loaded manifolds are promptly connected with the condenser and vacuum pump. When the pressure reaches 30 microns of mercury or less (usually within 3 minutes), the electrical refrigeration of the compartments is cut off. At this point room temperature air is blown into the compartments to elevate the temperature of the ampules and to hasten drying. In a typical run, the pressure gradually decreases to 1 micron or less during the next 8 to 12 hours. After approximately 24 hours, the line to the vacuum pump is closed and super-dry oil-pumped nitrogen (moisture less than 0.002 percent) is introduced into the evacuated system until atmospheric pressure is reached. The nitrogen-filled manifolds are clamped

and removed from the machine and the ampules are sealed with a gas-oxygen torch.

The Stokes drier used in this laboratory is equipped with a dry ice-alcohol condenser and is designed to permit refrigeration of the chamber by circulating cold alcohol through a surrounding jacket. In the present work the alcohol in the jacket is allowed to remain at room temperature.

Bottles of antigen to be dried are chilled in dry ice for 1 hour, rapidly transferred to metal trays, and loaded into the drier. Two pumps are used for initial evacuation of the chamber and when the pressure reaches 50 microns of mercury or less (within 30–60 minutes), one of the pumps is clamped off. In a typical run, the pressure gradually decreases to 10 microns in 6–10 hours and to as little as 2 microns in 24 hours. At the end of 24 hours, the line to the pump is closed and the chamber is brought to atmospheric pressure with super dry nitrogen. The trays of nitrogen-filled bottles are transferred to a hood which is pressurized with super dry nitrogen. Specially dried [2] sterile rubber closures (Corkage S23W, T. C. Wheaton Co., Millville, N. J.) are inserted into the bottles and fastened with triple aluminum seals (Wheaton—20 mm.) using a Wheaton "Roto-Seal" crimping machine (model No. 137). Finally, the seals are thoroughly coated with melted wax (No. 12, Dewey and Almy Chemical Co., Cambridge, Mass.) which provides added protection against moisture.

Hemagglutination Methods

The hemagglutination and hemagglutination-inhibition titrations are performed by a technique which conforms to the standard diagnostic method (*2*) recommended by the Committee on Standard Serological Procedures in Influenza Studies of the Armed Forces Epidemiological Board.

The ampule of dried antigen to be tested is rehydrated with 5.0 cc. of sterile distilled water, care being taken to incorporate all the dried material in the solution. One cc. of the rehydrated antigen is diluted with 19 cc. of physiological saline solution giving a 1:20 dilution of antigen and allowed to stand for 1 hour at room temperature; this facilitates elution of virus from the small amount of particulate matter in the preparation.

To determine the hemagglutinin titer, 0.5 cc. volumes of serial twofold dilutions of antigen in physiological saline solution are mixed in Kahn tubes with 0.5 cc. volumes of a 0.5-percent suspension of

[2] We are indebted to Mrs. B. Towne, E. R. Squibb & Sons, for the general method for preparing the rubber closures. The corkages are washed in Calgonite solution (Calgon Inc., Pittsburgh, Pa.), thoroughly rinsed with tap and distilled water and dried. Groups of 50 stoppers, wrapped in brown paper, are autoclaved at 15 lbs. pressure for 30 minutes. They are then dried for 3 hours at 100° C. over fresh phosphorus pentoxide in a desiccator which is evacuated to 100 microns of mercury or less through a dry ice-alcohol condenser. After drying, the stoppers are stored at room temperature over fresh phosphorus pentoxide.

human red blood cells (type "O"). The pattern of sedimented cells is read after the tubes have remained for 60 to 70 minutes at room temperature. The titer is the highest initial dilution of antigen giving complete hemagglutination.

The agglutination-inhibition titer of serum is determined as follows: The ampule of dried serum is rehydrated with 5.0 cc. of 1:10,000 Merthiolate (Lilly) giving a 1:10 dilution of the original serum. A portion of this is diluted tenfold ($\frac{1}{100}$ of undiluted serum) and 0.25 cc. volumes of serial twofold dilutions of serum are prepared in physiological saline solution. Four units (0.25 cc.) of the desired viral antigen and 0.5 cc. of a 0.5-percent human (type "O") red cell suspension are added to each tube of serum diluted in the range from 1:100 to 1:6400. The pattern of sedimented cells is read after the tubes have remained at room temperature for 55 to 60 minutes; the titer is the highest initial dilution of serum giving complete inhibition of hemagglutination.

Control Tests on Antigens and Antisera

Sterility Tests

Four ampules of dried antigen from each lot are rehydrated with 5.0 cc. volumes of sterile distilled water. One cc. amounts of the rehydrated contents are inoculated in quadruplicate in standard National Institutes of Health fluid thioglycollate medium (*4*). One set of duplicate tests is incubated at 35°–37° C. and the other at approximately 22° C. Two vials of antiserum from each lot are reconstituted with 5.0 cc. amounts of sterile physiological saline solution and tested as described above. Failure of the media to show growth after 10 days' incubation is considered a satisfactory test for sterility.

Tests for Potency and Specificity

These tests are performed on portions of the antigens and antisera rehydrated for sterility testing above. Antigens titering 1:160 or greater (based on the 5.0 cc. volume of allantoic fluid before drying) and antisera with inhibitory titers of 1:800 or more (based on initial volume of 0.5 cc. prior to drying) when tested with homologous antigen are of satisfactory potency. Each antigen is tested for specificity in agglutination-inhibition tests with PR8, FM1, and Lee antisera, and each serum is tested with all three antigens. The PR8 and FM1 antisera when diluted 1:100 or greater should not inhibit Lee antigen nor should Lee antiserum inhibit PR8 or FM1 antigens. Further, the PR8 antiserum should titer at least eightfold higher when tested with PR8 antigen than with FM1 antigen and vice versa.

Residual Moisture Determinations

Estimations of residual moisture are made on the contents of three separate ampules of antigen selected at random from each lot. Weighing bottles, with inside diameter of 20 mm. or greater and weighing less than 15 gm., are dried at 100°–150° C. in an oven and cooled over fresh phosphorus pentoxide in a desiccator. The bottles are weighed before and after addition of the dried antigen and the weight of sample (45–60 mg.) determined. The residual moisture value is calculated on the basis of weight loss of the sample following exposure at room temperature to fresh phosphorus pentoxide in a desiccator at a pressure of 150–200 microns of mercury for 24 to 30 hours. A balance with five-place accuracy is used for weighing.

Experimental Studies on Stability

Stability of Antigen on Drying and Storage at 4° C.

Table 1 summarizes the results of tests to determine the stability of 21 lots of antigen dried on the Bauer and Pickels machine in glass-seal ampules and stored at 4° C. for periods up to 25 months. The

Table 1. *Stability of dried influenza A and B hemagglutinating antigens on desiccation and storage at 4° C.*

Virus strain	Lot No.	Date dried	Titer [1]		Storage at 4° C.	
			Before drying	After drying	Period (months)	Titer
PR8	1A	August 1948	320	[2] 320	25	160
	1B	do	320	320	25	320
	3A	July 1949	640	320	13	320
	3B	do	640	320	13	640
	4A	August 1949	640	320	13	640
	4B	do	640	320	13	320
	5	April 1950	640	640	5	640
FM1	1A	August 1948	640	640	25	320
	1B	do	640	640	25	320
	3B	July 1949	320	160	13	320
	4B	August 1949	320	320	12	320
	5B	do	320	320	12	320
	6B	do	320	320	12	640
	9	April 1950	1,280	640	5	640
Lee	1A	August 1948	640	640	25	640
	1B	do	640	640	25	640
	4A	July 1949	640	320	14	640
	4B	do	640	320	14	640
	5A	August 1949	640	320	13	640
	6A	September 1949	640	640	11	1,280
	7	April 1950	640	640	5	1,280

[1] Tested within 1 week following desiccation.
[2] Numbers are reciprocals of hemagglutinin titers.

antigen was not appreciably reduced in titer on desiccation, nor was the potency of the dried materials significantly reduced on prolonged storage at refrigerator temperature.

Residual Moisture and Stability of Lyophilized Antigens on Heating

The data presented in table 2 revealed that the residual moisture values of the dried antigens ranged from 0.21 percent to 1.68 percent. All lots (PR8: 5, 11B, 11S, 12B, and 12; FM1: 9; and Lee: 7) with moisture values of 0.80 percent or less were dried in the Bauer and Pickels, or Stokes type equipment, by the procedures described earlier in this report; PR8: 4B, FM1:6A, and Lee: 6B were not. With the latter materials (which were dried on the Bauer and Pickels machine), the temperature of the refrigerated compartments of the drying apparatus was slowly elevated to room temperature over a period of 12 to 14 hours after beginning the procedure; this resulted in less thorough dehydration of the antigens which had residual moisture contents of 0.88 to 1.68 percent.

Table 2. *Stability of desiccated influenza hemagglutinating antigens on heating*

Strain	Lot No.	Drying		Moisture content [1] (percent)	Control titer [2]	Titer after treatment				
		Apparatus	Closure			Vial	37° C. 2 wks.	37° C. 7 wks.	56° C. 4 days	60° C. 2 days
PR8	5	B & P [3]	Glass seal	0.21	640	1	640	640	640	320
						2	320	ND	1,280	ND
	11B	B & P	do	.32	640	1	[4] ND	ND	640	320
						2	ND	ND	640	320
	11S	Stokes [5]	Rubber	.61	640	1	ND	ND	640	320
						2	ND	ND	640	320
	12B	B & P	Glass seal	.68	640	1	ND	ND	640	320
						2	ND	ND	640	320
	12S	Stokes	Rubber	.80	640	1	ND	ND	640	320
						2	ND	ND	640	320
	4B	B & P	Glass seal	1.68	640	1	320	ND	0	0
						2	640	ND	0	0
FM1	9	B & P	do	.32	640	1	320	640	1,280	640
						2	640	ND	640	640
	6A	B & P	do	.88	320	1	320	ND	0	0
						2	320	ND	0	0
Lee	7	B & P	do	.58	640	1	320	640	160	0
						2	640	ND	160	0
	6B	B & P	do	1.62	1,280	1	640	ND	0	0
						2	640	ND	0	0

[1] Average value of 2 to 4 ampules tested.
[2] Samples held at 4° C.
[3] B & P=Bauer and Pickels apparatus (*5*).
[4] ND=Not done.
[5] Stokes=Stokes apparatus (*7*).

The stability of the dried antigens on heating was related to the moisture value. With the exception of Lee lot 7, all antigens with values of 0.80 percent or less were stable at all the storage temperatures tested, while those with residual moistures of 0.88 percent or greater were completely inactivated on heating at 56° C. or 60° C. Lee lot 7, in which the moisture figure was only 0.58 percent, was partially inactivated at 56° C. and completely at 60° C. All the antigens tested, irrespective of moisture content, were stable for at least 2 weeks at 37° C.

960885—51——2

The adaptation of the chamber freeze-drying method to the production of stable influenza antigens represents some advance over the manifold drying technique heretofore employed in that it permits the use of bottle-type containers which can be sealed with rubber closures. While the over-all labor consumption in the filling, loading, and sealing of glass-seal ampules is roughly equal to that involved in processing the rubber closure bottles, the cost of container material in the latter instance represents only a small fraction of the former. In addition, the rehydration of antigen in the rubber sealed bottle is simple, and reconstituted material can be stored in the same vessel.

Stability of Rehydrated Antigens

The hemagglutinin in the rehydrated preparations of antigen was relatively stable (table 3), but, in order to obtain consistent titration endpoints with materials which were stored at 4° C. after rehydration, certain precautions were necessary. Precipitates appeared within a few days in the rehydrated antigens and increased progressively over a period of weeks. This phenomenon was more striking in reconstituted antigens than in the virus preparations which were maintained in the fluid state from the time of harvest. Reproducible results were obtained with such stored solutions only when the virus was eluted from the precipitated material. This was accomplished by dispersing the flocculated material uniformly in the solution, then removing a sufficient amount for the day's work, diluting 1:20 with physiological saline solution, and allowing the diluted material to stand at room temperature for 2 hours. When prepared in this manner, the resultant solutions contained as much hemagglutinin after storage of the stock antigen for 7 weeks at 4° C. as they did when initially rehydrated;

Table 3. *Stability of desiccated influenza A and B hemagglutinating antigens following rehydration*

Strain	Lot No.	Titer of rehydrated material stored at					
		4° C.				−20° C.	
		0 days	9 days	23 days	52 days	16 days	52 days
PR8	1A	160	320	160	160	----	----
	3A	320	----	----	----	320	320
	3B	640	1,280	640	640	----	----
	4B	320	----	----	----	160	320
	5	640	640	320	320	----	----
FM1	1B	320	320	160	160	----	----
	3B	320	----	----	----	320	160
	4B	320	160	160	160	----	----
	6B	640	----	----	----	320	320
	9	640	320	320	320	----	----
Lee	1A	640	640	640	640	----	----
	4A	640	----	----	----	640	640
	5A	640	1,280	640	640	----	----
	6A	1,280	----	----	----	320	640
	7	1,280	1,280	640	640	----	----

moreover, the specificity of the hemagglutinin was maintained. Rehydrated antigens which were refrozen and stored at −20° C. did not develop the precipitates mentioned above and their hemagglutinin content remained undiminished for 2 months.

Stability of Lyophilized Rooster Antiserum

Tests performed on 13 pools of lyophilized rooster antisera revealed no loss in titer of materials following drying and storage at 4° C. for periods up to 2 years.

Summary and Conclusions

A method for preparing dried antigens and control antisera for the agglutination-inhibition test for viral influenza has been described. Both manifold- and chamber-type drying equipment are satisfactory for this purpose. The hemagglutinating antigens show no more than twofold reduction in titer on desiccation. Properly dried antigens are stable for at least 25 months when stored at 4° C. and for at least 51 days at 37° C. Antigens with moisture values as high as 0.80 percent can usually be heated at 56° C. for 4 days or at 60° C. for 2 days without significant loss in titer while preparations with 0.88 percent or greater moisture content are completely inactivated under these conditions. Dried rooster control antisera are stable for at least 2 years when stored at 4° C.

REFERENCES

(*1*) van Rooyen, C. E., and Rhodes, A. J.: Virus Diseases of Man. Thomas Nelson and Sons, New York, 1948, p. 640.
(*2*) Committee on Standard Serological Procedures in Influenza Studies: An agglutination-inhibition test proposed as a standard of reference in influenza diagnostic studies. J. Immunol. **65:** 347–353 (1950).
(*3*) American Type Culture Collection. Viral and Rickettsial Registry. Washington, D. C., 1950, pp. 3, 4.
(*4*) National Institutes of Health: Culture media for the sterility test. Circular, 2d revision, dated February 5, 1946.
(*5*) Bauer, J. H., and Pickels, E. G.: Apparatus for freezing and drying virus in large quantities under uniform conditions. J. Exper. Med. **71:** 81–88 (1940).
(*6*) Smadel, J. E., Randall, R., and Warren, J.: Preparation of Japanese encephalitis vaccine. Bull. U. S. Army Med. Dept. **7:** 963–972 (1947).
(*7*) Flosdorf, E. W.: Freeze-drying (Drying by Sublimation). Reinhold Publishing Corp., New York, 1949, Chapter 5.

A Yolk Sac Technique for the Routine Isolation of *Brucella*

Injection of Clotted Blood Specimens Into Embryonating Eggs With Recovery of All Three Species

By KATHLEEN GAY, B. S., and S. R. DAMON, Ph. D.*

Efforts to recover *Brucella* from blood specimens submitted to a public health laboratory are often unsuccessful. Because of the apparent need of a more successful method adaptable to routine use, investigation of the embryonating egg as a possibly superior medium for the recovery of *Brucella* was undertaken. Various investigators, e. g., Goodpasture and Anderson (*1*), Buddingh and Womack (*2*), and Ruiz-Castaneda (*3*), had previously described this organism as preferentially an intracellular parasite; and Spink et al. (*4*), among others, had found the embryonating egg valuable in the study of therapeutic agents for the treatment of brucellosis. However, to our knowledge, no one had attempted to employ the egg on a large scale in the isolation of *Brucella*.

A preliminary experiment (*5*) indicated that the yolk sacs of 3- to 6-day-old embryos provided a highly nutritive medium for multiplication of *Brucella*, even though only a few organisms were present, and that this age range was probably optimum for the inoculation of blood.

This paper presents the investigation of a yolk sac technique and the results of its application. Embryos 3 to 5 days old were employed for the inoculation of clotted blood specimens. All three species of *Brucella* were recovered.

Materials and Methods

Specimens

Clots from specimens of blood received routinely at the Indiana State Board of Health for diagnostic agglutination tests were employed. Following completion of the serological test, one-half of the clot was forced through a 5 ml. syringe, without the needle, into a tube of crystal violet tryptose broth for routine culture. The other half was then prepared for animal and egg inoculations by expelling

*Senior Bacteriologist, and Director, respectively, Bureau of Laboratories, Indiana State Board of Health, Indianapolis. This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service, Bethesda, Md.

it into a bottle containing glass beads and 5–7 ml. of tryptose broth. The bottles were stoppered and shaken 3 minutes for further disintegration of the clots.

Eggs

White Leghorn "hatching quality" eggs (av. wt. 59 gm.) were obtained weekly from a local poultry supply house and held at 20° C. until the day of incubation. That is, a week's supply was received on Wednesday and a sufficient number incubated each Monday and Friday, so that each day of the week 3-, 4-, or 5-day old live embryos were available. Before and after injection, all eggs were kept at 98°–99° F. (±37° C.), relative humidity 50°–56°, in an electric hatchery incubator. They were not turned at any time, since doing so was found nonessential to the technique and complicated the procedure for locating the yolk sacs.

Inoculation of Eggs

Eggs which had incubated 3 to 5 days were candled, using a 100-watt bulb installed in a plywood box 20″ square. The interior of the box was painted black and one side left entirely open to receive the egg racks. The opening was covered with a photographer's heavy black cloth. With this device it was possible to candle accurately embryos as young as 3 days, mark the position of the embryos, and select the live ones for inoculation.

A small hole was drilled into the large end of the egg with an electric table model dental drill and No. 22 S. S. White abrasive point. With a 20-gage, 1½-inch hypodermic needle, 0.5 ml. of the blood-broth specimen was injected into the yolk sac. In making the inoculation the egg, with the marked embryo at the top, was placed horizontally on the table and the needle inserted toward the center for 0.5–0.75 inch. If the embryo was 3 days old, the direction of the needle was slightly upward; if 4- or 5-days, directly inward toward the center. The area around each hole was swabbed before and after inoculation with tincture of iodine, and the hole finally sealed with a swab of collodion.

Examination of Eggs

Examinations consisted of two or three subcultures from the yolk at about 4- or 5-day intervals and in some instances a final harvesting of all live embryos on the 18th or 19th day of total incubation, the embryo ages at the three subculture periods being 9–10, 14, and 18–19 days, respectively.

The yolk was withdrawn in a manner similar to that of the injections. However, since the position of the yolk sac gradually changes with further development of the embryo, allowances must be made

for this by inserting the hypodermic needle at different angles and depths. As a rule, the older the embryo the more the yolk position shifts from a high anterior position to a low posterior one. The chart explains diagrammatically the method for withdrawing yolk from the different age embryos.

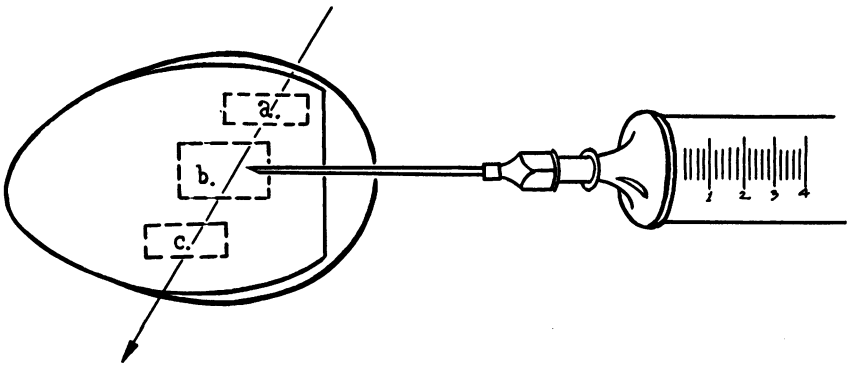

Diagram of the relative positions of the yolk sac as the embryo develops. Each rectangle represents the approximate center of the yolk sac at (a) 3 days, (b) 4–5, 9–10 days, and (c) 14 days of age.

Several drops of yolk material withdrawn from each egg were streaked over the surface of a slightly dehydrated tryptose agar (pH 6.6–6.7) plate. Those embryos remaining viable on the 18th or 19th day of incubation were harvested and portions of the blood, yolk sac wall, internal organs, and embryo fluids streaked onto tryptose agar plates. All plates, before being discarded as negative, were held 2 weeks in an incubator equipped to maintain a CO_2 concentration of approximately 10 percent.

C-V Enrichment Technique

The broth employed was crystal violet tryptose broth (modification of a Huddleson formula) routinely used in this laboratory for the isolation of *Brucella*. The final dye concentration was 1:1,000,000. Following inoculation of the clot, the tubes of broth were placed in the CO_2 incubator; and at the end of each week, for a period of 3 weeks, the broth was subcultured to a plain tryptose agar plate and a tryptose agar plate with crystal violet added to a final concentration of 1:1,000,000. Plates were incubated as described above.

Guinea Pig Inoculation

The clot was treated for guinea pig inoculation in the same manner as for egg injections, except that each guinea pig received 1–1.5 ml. per pig subcutaneously in the groin region whereas each egg received

only 0.5 ml. Two pigs per specimen were inoculated. They were sacrificed at the end of one month and the internal organs cultured. An agglutination test was also performed on each pig.

Identification of Brucella

Suspicious colonies on plates were fished to tryptose agar slants and incubated in the CO_2 incubator for 24–48 hours. A spot agglutination test was then made from the growth on the slant, using *Br. abortus* antiserum. If clumping occurred, duplicate tryptose agar slants were inoculated, one incubated in air and one in CO_2 to check growth requirements. A saturated lead acetate paper was placed in the mouth of the latter to determine H_2S production. Growth from the original slant was also streaked onto basic fuchsin and thionin dye plates (dye concentration 1:100,000) for species typing. The remaining growth was then suspended in 0.5 percent formalinized saline, diluted to the proper density, and tube agglutination tests set up with both the *Br. abortus* antiserum and the homologous serum from the patient.

Experimental Results

The first specimens of blood injected into eggs were clots from the heart blood of autopsied guinea pigs, which had been inoculated subcutaneously with human blood from suspected cases of brucellosis, and clotted peripheral blood from swine experimentally infected with *Brucella suis*. Fifteen specimens were injected into 3- and 5-day-old embryonating eggs and two yolk subcultures made. Four *Br. suis* strains were recovered, one from the guinea pig and three from the swine bloods. About the same time a *Br. melitensis* type was isolated from the blood of a treated ambulatory case when the clot was inoculated into the yolk sacs of 3-day-old embryos. This latter isolation was not made from either the crystal violet tryptose broth or the guinea pigs, although the clot was examined by both these methods. Following the success with these preliminary specimens, it was decided to continue the investigation, employing clots from specimens received routinely for diagnostic agglutination tests.

Experiment 1. Twenty-seven human clots from sera with varying agglutination titers were inoculated into each of six eggs and into a tube of C-V tryptose broth, as described under Materials and Methods. Fifteen of the clots were further examined by injection into guinea pigs. Seventeen sets of the eggs receiving the blood clot specimen also received penicillin G in three of the six eggs in an attempt to control the overgrowth of contaminants. Examinations consisted of two weekly yolk subcultures.

Three of the clots inoculated proved heavily contaminated when

injected into the embryos, even though penicillin was used, and the eggs were not examined. The 24 satisfactory examinations yielded two *Br. abortus* strains. No recoveries were made from either the C-V broth or the guinea pigs, although both mediums received a portion of the clots which yielded these two isolations.

Experiment 2. The specimens in this group were of the same nature as those employed in experiment 1. However, the clot-broth mixture was inoculated into four eggs each and penicillin was not used. Fourteen clots were injected into eggs and C-V tryptose broth, with eight of these also being examined by the guinea pig inoculation technique.

One *Br. abortus* strain was recovered from the chick embryo yolk but not from the C–V tryptose broth nor the inoculated guinea pigs. No additional recoveries were made by either of the last two methods. One blood clot, because of contamination, was considered unsatisfactory for egg inoculation.

Experiment 3. The specimens comprising this group came from patients whose sera exhibited a complete agglutination at a 1:20 or higher serum dilution level when the test was made with a rapid slide antigen. Materials and techniques used throughout this experiment were described in the foregoing section. A total of 81 specimens were injected into the embryo yolk sacs. All the clots were simultaneously examined by inoculation into C-V broth; and 71 of them, by injection into guinea pigs. Those eggs inoculated with 41 of the specimens were examined a third time, as previously described.

When injected into eggs, 23 clots resulted in a heavy contamination which rapidly killed all embryos and were considered unsatisfactory for egg examinations. From the remaining 58 satisfactory sets of eggs, nine recoveries, six *abortus* and three *suis* strains, were made. None of these were recovered from the guinea pigs, although all except one of the specimens yielding an isolation were injected into the pigs; and only five of them were obtained from the incubated C-V broth.

From the entire group of specimens included in this experiment two additional isolations were made: an *abortus* strain from the C-V broth only, this particular specimen being unsatisfactory for egg examination; and a *suis* strain recovered from both the guinea pig and the C-V broth, with the chick embryos failing to yield the isolation. In those instances in which the yolks were examined for a third time or the embryos harvested, no additional recoveries were made.

Summary of Experiments. To summarize, a total of 138 blood clot specimens, of which 123 were from human sources, were inoculated into the embryonating egg. Four *suis* strains were recovered from the 15 lower animal clots included in the study.

The 123 human clots were further examined by enrichment in C-V

broth and 95 of them by guinea pig inoculation. Twenty-seven clots, upon injection into the eggs, resulted in a heavy contamination and rapid death of the embryos. Thus, only 96 specimens were considered satisfactory for egg inoculations, and from this number 14 recoveries—1 *melitensis*, 4 *suis*, and 9 *abortus*—were made, with the egg technique yielding all but one *suis* strain. From these same 96 specimens the guinea pigs yielded one *Brucella* strain and the C-V broth, six. Data concerning these clots are presented in table 1. Eleven isolations came from patients exhibiting acute symptoms at the time of collecting the specimens, while the other three were from asymptomatic cases. No recoveries were made from patients with chronic complaints. Also, all the isolations came from persons with agglutination titers of at least a 1:80 serum dilution.

Table 2 summarizes the total recoveries made from blood clots (inclusive of the guinea pig and swine bloods) inoculated into the

Table 1. *Data relative to specimens examined by the egg inoculation technique*

Symptomatology of patients at time of collecting specimens

	Acute	Chronic	Asymptomatic		No information
			Treated	Untreated	
Number	41	28	17	4	6
Isolations	11	0	1	2	0

Agglutination titer [1] of sera from specimens

	1:20	1:20–1:80	1:80–1:320	1:320–1:640	1:640
Number	5	8	48	21	14
Isolations	0	0	7	5	2

[1] The highest serum dilution in which a 2+ agglutination reading was obtained.

Table 2. *The recovery of* Brucella *from blood clots examined by the yolk sac technique*

Type	Laboratory No.	Titer [1]	Specimen source	Age of embryo: days	
				At injection	At isolation
	2		Swine	5	13
	12		do	5	13
	19		do	5	10
Suis	5973		Guinea pig	3	8
	4612	1:80	Human	5	14
	4621	1:80	do	3	8
	4626	1:640	do	3	13
Melitensis	6356	1:320	do	3	12
	3338	1:320	do	4	9
	3953	1:160	do	4	10
	4046	1:80	do	3	10
	4138	1:320	do	4	9
Abortus	2172	1:160	do	4	9
	4712	1:320	do	5	10
	4776	1:320	do	3	10
	1403	1:160	do	3	8
	1575	1:160	do	3	8

[1] The highest serum dilution in which a 2+ agglutination reading was obtained.

960885—51——3

yolk sacs of 3- to 5-day embryonating eggs. The age of the embryo at the time of injection of the clot and also at the time of recovery of the strain is included. The age given for the time of isolation is that age of the first embryo to yield an isolation on the day the yolk was withdrawn. Primary isolations have been made from all three ages employed, although not all eggs inoculated from any one specimen always yielded a simultaneous isolation. Nearly all recoveries were obtained by the eighth day of incubation of the injected specimens; and no doubt the others would have been if the yolk withdrawal had occurred at that time.

Discussion

It is not the intention of the authors to suggest that the specimens employed in this study are the most suitable for the recovery of *Brucella* from suspected cases of brucellosis. However, they are the type most frequently submitted by a physician who desires a laboratory diagnosis, usually a determination of the agglutination titer; and certainly a test which permits isolation of the causative organism from the same specimen used in performing an agglutination test would be highly desirable.

Also, it should be mentioned that the percentage of isolations from these specimens has been decidedly influenced by two limiting factors. First, the amount injected into each egg was actually the equivalent of only one-fourth of 0.5–1.5 ml. of whole blood. This amount is extremely small when compared with the amount of blood considered desirable for culturing procedures. Second, although the specimens usually came from a patient showing a fairly high agglutination titer, as shown in table 1, we find that less than 50 percent came from persons exhibiting acute symptoms. Many clots from treated cases and from persons with chronic complaints were included in the series. Taking into consideration these factors, the egg embryo yolk sac technique appears to be an efficient as well as a comparatively rapid means of isolating *Brucella* from clotted blood specimens.

Summary

1. A method for using the embryonating chick egg for primary isolation of *Brucella* from the blood stream has been presented and supporting experimental data given.

2. Essentially, this technique consists of injecting specimens directly into the yolk sacs of 3-, 4-, and 5-day-old embryos and subculturing the yolk periodically.

3. Seventeen recoveries, representing all three species of *Brucella* have been made from clotted blood specimens injected into the embryo yolk sacs.

4. The egg embryo yolk sac technique is recommended because: (*a*) it provides living tissue cells in a compact medium; (*b*) it permits recovery of all three types of *Brucella*; (*c*) it gives comparatively quick results; and (*d*) it entails a minimum amount of danger for laboratory personnel.

ACKNOWLEDGMENT

The authors are indebted to the Department of Veterinary Science, Agricultural Experiment Station, Purdue University, for the guinea pig and swine clotted blood specimens employed in the initiation of this study; and for the provision, maintenance, and examination of all guinea pigs to which reference has been made. The data resulting from the examinations is extracted from another phase of the joint brucellosis project and is included in this paper only inasmuch as it pertains to specimens inoculated into embryonating eggs.

Since submission of the manuscript for publication the authors have received a communication from Dr. Felix E. Ramacciotti of the Faculty of Medicine, Cordoba University, Cordoba, Argentina, describing the successful use of the chick embryo in recovering *Brucella* from the blood stream of 10 patients. De la Revista de la Facultad de C. Médicas de Córdoba, vol. 9, No. 1, 1951.

REFERENCES

(*1*) Goodpasture, E. W., and Anderson, K.: The problem of infection as presented by bacterial invasion of the chorio-allantoic membrane in chick embryos. Am. J. Path. **13**: 149 (1937).

(*2*) Buddingh, G. J., and Womack, F. C.: Observations on the infection of chick embryos with *Bacterium tularense*, *Brucella*, and *Pasteurella pestis*. J. Exper. Med. **74**: 213 (1941).

(*3*) Ruiz-Castaneda, M.: Studies on the pathogenesis of brucellosis. Proc. Soc. Exper. Biol. and Med. **64**: 298 (1947).

(*4*) Spink, W. W., Hall, W. H., Shaffer, J. M., and Braude, A. I.: Human brucellosis. J. A. M. A. **136**: 382 (1948).

(*5*) Gay, Kathleen, and Damon, S. R.: Use of the chick embryo for isolation of *Brucella*. Multiplication of the organism in the yolk sac and selection of the embryo age optimal for isolation from blood. Pub. Health Rep. **65**: 1187 (1950).

A Modification of the Winter and Sandholzer Media and Technique for Enterococci Detection

By WEN-LAN LOU WANG, M.S., and STUART G. DUNLOP, PH.D.*

The use of fecal streptococci as an indicator of pollution of water has been proposed by a number of investigators (*1–5*). Also, various media and techniques have been suggested for the detection of these organisms (*4, 6–11*). In the course of experiments conducted on a Public Health Service research project in this laboratory, poor confirmation of presumptive positive counts from sewage and irrigation water was obtained, when the media proposed by Winter and Sandholzer (*10*) were used for the estimation of enterococci. The present investigation was undertaken to determine the factor or factors causing the poor confirmation and, if possible, to suggest methods of overcoming the difficulties encountered.

Materials and Methods

The method described by Winter and Sandholzer consisted of two parts. The production of acid and growth turbidity in a sodium azide enrichment medium after incubation at 45° C. was interpreted as presumptive evidence of the presence of enterococci. Positive presumptive tubes were then confirmed by inoculating a slant-broth preparation of a penicillin-sodium azide medium. Pin-point colonies on the slant, growth sediment in the broth, the presence of gram-positive ovoid streptococci in the broth, and a negative catalase test constituted a confirmed positive.

In the preliminary experiments in this study, the technique and media proposed by Winter and Sandholzer were employed, except that the positive presumptive tubes were not transferred to the confirmation medium until after 18 to 20 hours' incubation. Winter and Sandholzer recommended that the transfers be made as soon after 8 hours as the tubes became positive. Duplicate tubes of the presumptive medium were inoculated with sewage-polluted irrigation water collected locally, using at least three dilutions for each sample; and counts were computed in terms of the most probable number

*Graduate Assistant, and Assistant Professor, Department of Bacteriology,, University of Colorado School of Medicine, Denver. This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service. The material in this paper was taken in part from the M. S. thesis of Wen-Lan Lou Wang, University of Colorado.

(MPN), as specified for the coliform group of organisms in Standard Methods for the Examination of Water and Sewage (*12*). The pH of each positive presumptive tube was determined with a Beckman pH meter at the time of transfer. In addition to the Winter and Sandholzer confirmation technique, a loopful of each presumptive positive growth was streaked on a blood agar plate, and the organisms isolated were tested for their ability to grow in 6.5-percent sodium chloride broth and on 40-percent bile agar, and by the reduction of litmus milk and 0.1-percent methylene blue milk. This procedure was likewise carried out with the confirmed growths.

It was noted in the early experiments that better confirmation results were obtained if one drop of the sediment of the positive presumptive growth was transferred to the confirmation medium rather than only one loopful of the culture, as suggested by Winter and Sandholzer. Also, when 10 ml. quantities of sample were required, 10 ml. of double strength presumptive medium were employed rather than 2 ml. of fivefold strength medium, since the latter frequently showed a positive pH change simply on addition of the sample.

After studies were made of the effect of the 18- to 20-hour incubation time, as compared with 10 to 24 hours, modifications of the original presumptive medium of Winter and Sandholzer were tried in an effort to retard the speed of the pH change to permit use of overnight incubation. Experiments were conducted to compare these modifications with the original medium of Winter and Sandholzer (WS) and the *Streptococcus fecalis* (SF) medium of Hajna and Perry (*7*). Of the various modifications tried, the following formula gave the most satisfactory results:

	Percent
Tryptone (Difco)	0. 7
Tryptose (Difco)	. 3
K_2HPO_4	. 4
Glucose	. 5
Yeast extract (Difco)	. 5
Sodium azide	. 04
Brom thymol blue	. 0032

Experimental Studies and Discussions

Preliminary experiments indicated that both the time and temperature of incubation of the presumptive positives might affect the results of confirmation. Table 1 shows that poor confirmation resulted when the presumptive positives (growing in original WS presumptive medium) were allowed to incubate overnight (20 hours); better confirmation resulted if the presumptive positives were transferred to the confirmation medium as soon as the tubes became positive. In exper-

Table 1. *The effect of incubation time on the confirmation of positive presumptive tubes*

Experiment No.	Presumptive test [1]			Confirmation tests [2]			
	Incubation at 45° C. (hours)	MPN count per 100 ml.	pH range positives	Incubation at 37° C. (hours)	MPN count per 100 ml. WS medium	Blood plates	WS medium
Va	20	2,300	4.99–6.57	24	230	[3] 4/6	[3] 2/6
Vb	10–24	2,300	5.11–6.57	24	2,300	6/6	6/6
VIa	20	6,200	4.30–6.04	24	62	6/7	3/7
VIb	10–24	6,200	4.96–6.04	24	1,300	6/7	5/7

[1] Original WS presumptive medium.
[2] Confirmation by blood plates included growth in 6.5-percent NaCl broth and on 40-percent bile agar, and reduction of litmus milk and 0.1-percent methylene blue milk. Confirmation by WS medium included pin-point colonies on the slant, growth sediment in the broth, the presence of gram-positive ovoid streptococci in the broth, and a negative catalase test.
[3] Ratio of confirmed positives to presumptive positives.

iment Vb, four more positive tubes were confirmed in the WS confirmation medium than in Va, and experiment VIb showed two more positive confirmed tubes than VIa. These differences were reflected likewise in the relative MPN counts. Also, definite differences in the quantity of growth could be seen on the blood plates inoculated from these presumptive tubes; much heavier growth resulted when the positives were transferred early. It is apparent, too, in this table, that there was less pH change in the cultures transferred soon after they became positive. Combination of the results of these two experiments shows that only 5 tubes out of 13, or 38 percent, were confirmed using the 20-hour incubation time; while 11 out of 13, or 85 percent, were confirmed when the presumptive positives were transferred to the confirmation medium as soon as the tubes became positive. This difference is statistically significant at the 5-percent level. Thus, it may be concluded that the delayed transfer of the growth from presumptive positives to the confirmation medium results in poor confirmation.

It has been demonstrated by Bagger (*13*) that enterococci have relatively high resistance to acidity and heat. Sherman (*14*), however, stated that *Streptococcus zymogenes* and *Streptococcus durans* had lower heat resistance than the other fecal streptococci. So it may be possible that the poor confirmation with the WS medium resulted from death or attenuation of the enterococci by the combination of high acidity and long incubation time at the 45° C. temperature.

Attempts were then made to protect the enterococci in the positive presumptive tubes by retarding the speed of the pH change. Table 2 shows the effect of modifying the WS presumptive medium with a slight increase in the protein content and by the addition of buffer in the form of K_2HPO_4. Included also is a comparison of these media with the SF medium of Hajna and Perry. This table indicates the modified WS medium gave slightly better confirmation than the

Table 2. *Effect of buffering WS presumptive medium and comparison with SF medium*

[Incubation of presumptives: 45° C. for 18 hours; incubation of confirmation tubes: 37° C. for 24 hours]

Experiment No.	Presumptive test			Confirmation tests [1]		
	Medium used	MPN count per 100 ml.	pH range positives	MPN count per 100 ml. WS medium	Blood plates	WS medium
VIII	WS	2,300	4.89–5.24	62	[2] 6/6	[2] 3/6
	WS+buffer and protein	2,300	6.02–6.24	2,300	6/6	6/6
IX	WS	620	4.77–5.26	230	3/3	2/3
	WS+buffer and protein	130	5.81–6.69	130	2/2	2/2
	SF	60	5.68	60	1/1	1/1
X	WS	2,300	5.02–6.08	620	6/6	5/6
	WS+buffer and protein	620	5.22–5.91	620	5/5	5/5
	SF	230	5.51–6.15	230	4/4	4/4

[1] Confirmation by blood plates included growth in 6.5-percent NaCl broth and on 40-percent bile agar, and reduction of litmus milk and 0.1-percent methylene blue milk. Confirmation by WS medium included pin-point colonies on the slant, growth sediment in the broth, the presence of gram positive ovoid streptococci in the broth, and a negative catalase test.

[2] Ratio of confirmed positives to presumptive positives.

original WS presumptive medium, and the pH changes were less. However, in general, lower presumptive MPN counts were obtained, that is, fewer tubes showed positive. Although in these experiments 10 tubes out of 15, or 67 percent, were confirmed from the WS presumptive medium compared with 13 out of 13, or 100 percent, from the modified WS medium, statistical evaluation of this difference indicates borderline significance (critical ratio=1.80; P=7.2 percent).

The SF medium is not significantly better than the original WS medium due, probably, to the small number of tubes involved in the calculations with the SF medium. SF medium contains greater amounts of sodium azide, protein, and phosphate buffer than either WS or modified WS medium, and is undoubtedly more inhibitory. Further, it was noted that occasionally tubes of the SF medium which did not show a change in the indicator could be demonstrated to contain enterococci. This would suggest that the pH range of brom cresol purple, 5.2 to 6.8, as used in SF medium, is too low to indicate light growth of the organisms. Brom thymol blue which was used in the WS and modified WS media has a pH range of 6.0 to 7.6. Mallmann and Seligmann (*11*) also noted that streptococci frequently were present in the tubes of SF broth when no visible color change of the indicator took place.

In the experiments thus far reported, the maximum incubation time of the presumptive media had been 20 hours. It was thought that continued observation of the negative tubes for an additional 28 hours might result in better presumptive counts. That this is so is shown in table 3. By prolonging the incubation time of the negative tubes, the modified WS medium and the SF medium showed presumptive MPN counts more comparable with those of the original WS medium, while still maintaining good confirmation. Combina-

Table 3. *Effect of prolonging incubation time of negative presumptive tubes*

[Incubation of presumptives: 45° C. for 18–48 hours; incubation of confirmation tubes: 37° C. for 24 hours]

Experiment No.	Presumptive test			Confirmation tests [1]		
	Medium used	MPN count per 100 ml.	pH range positives	MPN count per 100 ml. WS medium	Blood plates	WS medium
XI	WS	62,000	4.81–6.58	500	[2] 9/9	[2] 5/9
	WS+buffer and protein	23,000	5.47–6.37	23,000	8/8	8/8
	SF	620	5.28–6.29	620	5/5	5/5
XII	WS	62,000	4.95–5.88	5,000	8/9	6/9
	WS+buffer and protein	62,000	5.40–6.89	62,000	9/9	9/9
	SF	6,200	5.23–6.33	6,200	7/7	7/7
XIII	WS	23,000	4.92–6.98	6,200	7/8	6/8
	WS+buffer and protein	23,000	5.32–7.03	6,200	7/8	7/8
	SF	23,000	5.20–6.31	6,200	8/8	7/8

[1] Confirmation by blood plates included growth in 6.5-percent NaCl broth and on 40-percent bile agar, and reduction of litmus milk and 0.1-percent methylene blue milk. Confirmation by WS medium included pin-point colonies on the slant, growth sediment in the broth, the presence of gram-positive ovoid streptococci in the broth, and a negative catalase test.

[2] Ratio of confirmed positives to presumptive positives.

tion of the results of experiments XI, XII, and XIII revealed that enterococci were isolated on blood plates from nearly all the positive WS presumptive tubes (24 out of 26), but only 17 of the 26, or 65 percent, were confirmed in the WS confirmation medium. Twenty-four of the 25 positives, 96 percent, from the modified WS medium, and 19 of the 20 positives from the SF medium, 95 percent, were confirmed in the WS confirmation medium. The differences, comparing the confirmation results (in WS confirmation medium) from the modified WS medium and the SF medium with the results from the original WS presumptive medium, are statistically significant at the 5-percent level. Thus, by buffering the WS presumptive medium to retard the pH change of the positives, and by observing the negatives for an additional 28 hours, good confirmation and more comparable presumptive counts were obtained. The SF medium, however, still did not give as high counts as the WS or modified WS media.

In all the experiments the organisms isolated on blood plates from the confirmation tubes proved to be enterococci. On the other hand, gram-positive rods, diphtheroids, atypical enterococci (organisms failing to give characteristic reactions in all four tests used) and streptococci other than enterococci were isolated occasionally from the presumptive tubes. Mallmann and Seligmann (*11*) likewise found that gram-positive rods occasionally occurred in azide dextrose broth (Difco), azide broth (Mallmann *6*), and SF broth of Hajna and Perry (*7*) when these media were used to test the presence of streptococci in river water contaminated by sewage and in swimming pool water. The occurrence of atypical enterococci in swimming pool water was noted by Ritter and Treece (*5*).

Table 4 shows the combined confirmation results of experiments

Table 4. *Comparison of confirmation on blood plates and in WS confirmation medium*

[Combined results of experiments VIII through XIII]

Presumptive medium	Total positive presumptive tubes	Confirmation [1]			
		Blood plates		WS medium	
		Tubes positive	Percent	Tubes positive	Percent
WS	41	39	95	27	66
Modified WS	38	37	97	37	97
SF	25	25	100	24	96

[1] Confirmation by blood plates included growth in 6.5-percent NaCl broth and on 40-percent bile agar, and reduction of litmus milk and 0.1-percent methylene blue milk. Confirmation by WS medium included pin-point colonies on the slant, growth sediment in the broth, the presence of gram-positive ovoid streptococci in the broth, and a negative catalase test.

VIII through XIII. Comparison of the confirmation on blood plates indicates that there is little difference among the three media as to the validity of the observed presumptive positives, although it appears from this and the other tables that the SF medium gives lower counts than the other two media. A striking difference, however, is noted in the proportion of tubes confirmed in the WS confirmation medium, only 66 percent of the WS presumptive positives being confirmed, compared with 97 and 96 percent from the modified WS and SF media, respectively.

In the experiments already described, many organisms not capable of growing in the confirmation medium were isolated from positive WS presumptive tubes and later were identified as enterococci. On subsequent inoculation of six of these cultures into confirmation media, all showed positive confirmation. Thus, it might be suggested that the confirmation medium of Winter and Sandholzer required a rather heavy inoculum of healthy organisms. It was also noted that some of the confirmation tubes, negative after 24 hours, showed growth after 5 days of incubation, and enterococci were isolated. This might be explained by the probable inactivation of penicillin after several days' incubation at 37° C. Ostrolenk, Kramer, and Cleverdon (*4*) reported that the addition of 6.5-percent NaCl to SF medium reduced the most probable number counts of enterococci. White and Sherman (*9*) also showed that *Streptococcus durans* in milk was inhibited partially in the presence of 325 Oxford units of penicillin per liter. Accordingly, attempts were made to modify the confirmation medium by reducing the quantities of penicillin or NaCl. The results were suggestive of the fact that such modification would increase the proportion of confirmation. Apparently, good confirmation can be obtained by reducing the quantity of the broth containing the penicillin and NaCl and inoculating heavily with sediment from the presumptive positives.[1] However, if the Winter and Sandholzer presumptive

[1] Method used by the Environmental Health Center, Public Health Service, Cincinnati, Ohio.

medium is modified by the addition of buffer and by increasing the protein, good confirmation results can be obtained with the unmodified confirmation medium. Further work is in progress on the use of these and other media for the estimation of enterococci in vegetable washings.

Summary

Poor confirmation of presumptive positive counts of fecal streptococci from sewage and irrigation water had been obtained using the media proposed by Winter and Sandholzer. Studies on blood agar plates, followed by biochemical tests on the organisms isolated, indicated that enterococci were present in at least 95 percent of the presumptive positives tubes, even though only 66 percent would confirm in the Winter and Sandholzer confirmation medium. By buffering the Winter and Sandholzer presumptive medium to retard the pH change of the positives and by observing the negatives for an additional 28 hours, good confirmation and comparable presumptive counts were obtained.

REFERENCES

(*1*) Savage, W. C., and Read, W. T.: The significance of streptococci in water supplies. J. Hyg. **15:** 334–351 (1916).
(*2*) Mallmann, W. L.: Streptococcus as an indicator of swimming pool pollution. Am. J. Pub. Health **18:** 771–776 (1928).
(*3*) Thresh, John: The Examination of Water and Water Supplies, ed. 5. Edited by E. V. Suckling, Blakiston Co., Philadelphia, 1943, pp. 445–448, 503, 506.
(*4*) Ostrolenk, M., Kramer, N., and Cleverdon, R. C.: Studies of enterococci and *E. coli*. J. Bact. **51:** 197–203 (1947).
(*5*) Ritter, C., and Treece, L. E.: Sanitary significance of cocci in swimming pools. Am. J. Pub. Health **38:** 1532–1538 (1948).
(*6*) Mallmann, W. L.: A new yardstick for measuring sewage pollution. Sewage Works J. **12:** 875–878 (1940).
(*7*) Hajna, A. A., and Perry, C. A.: Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci. Am. J. Pub. Health **33:** 550–556 (1943).
(*8*) Chapman, G. H.: The isolation of streptococci from mixed cultures. J. Bact. **48:** 113–114 (1944).
(*9*) White, J. C., and Sherman, J. M.: Occurrence of enterococci in milk. J. Bact. **48:** 262 (1944).
(*10*) Winter, E. A., and Sandholzer, L. A.: Recommended procedure for detecting the presence of enterococci. Commercial Fisheries T. L. 2, Fish and Wildlife Service, U. S. Dept. of the Interior, Washington, D. C., 1946.
(*11*) Mallmann, W. L., and Seligmann, E. B.: A comparative study of media for the detection of streptococci in water and sewage. Am. J. Pub. Health **40:** 286–289 (1950).
(*12*) Standard Methods for the Examination of Water and Sewage, ed. 9. American Public Health Association, New York, 1946.
(*13*) Bagger, S. V.: The enterococci. J. Path, and Bact. **29:** 225–238 (1926).
(*14*) Sherman, J.: The streptococci. Bact. Rev. **1–2:** 1–87 (1937).

Incidence of Disease

No health department, State or local, can effectively prevent or control disease without knowledge of when, where, and under what conditions cases are occurring

UNITED STATES

Reports From States for Week Ended September 1, 1951

Malaria

In reply to a request for additional information on malaria throughout the United States, certain States have reported as follows on cases prior to the current week. Colorado received reports of 169 cases from a military hospital from June 30 to August 25. In Wisconsin, 126 cases, 102 of which were from one military establishment, were reported from January 1 to August 18. Of the 14 civilian cases, 8 are said to have been acquired outside the United States. Twenty-four cases have been reported in Maryland, exclusive of Baltimore, and of these, 23 contracted the infection in Korea, and the remaining 1 from another foreign country. Seven of the twenty-four are classified as civilian cases, but all are returned veterans. Baltimore has received reports of 8 cases in civilian seamen and a like number in military personnel in an Army hospital. Tennessee has reported 85 "Korean malaria cases." Two civilian cases reported recently, which were confirmed by laboratory examination, apparently were infected locally. One was a vivax infection and the other, quartan. It is suggested that the source of infection in the latter may have been mosquitoes which had fed on neurosyphilitic patients who had received treatment. A total of 412 cases among civilians was reported in Texas for the 34 weeks ended August 25, and 114 were reported from military establishments for the 15 weeks ended August 25. Nebraska; Boston, Mass.; and New Orleans, La., reported a few cases each, and Nevada reported none for the past 8 months.

For the current week, each of 24 States reported 1 or more cases of malaria. A total of 127 cases from military establishments and of 55 in civilians was reported. A certain proportion of the latter may have been persons with previous military service. States reporting more than 10 cases were: Missouri, 29; Wisconsin, 25; Oklahoma, 25; Texas, 23; Colorado, 13; and Illinois, 11.

Poliomyelitis

A total of 1,742 cases of poliomyelitis was reported for the current week as compared with 1,770 last week. This suggests that the peak of incidence has been reached for the country as a whole, which is

approximately the same as it was in 1949. In 1950 the peak week was much later than usual—late in September.

For the current week incidence decreased, as compared with the previous week, in the New England, West North Central, East South Central, West South Central, and in the Mountain States. Only slight increases over the previous week occurred in the other parts of the country.

The cumulative total of poliomyelitis cases for the calendar year is 13,632 as compared with 13,480 for the same period in 1950. Since the seasonal low week the cumulative total is 12,420, which is approximately the same as in 1950 (12,349).

Epidemiological Reports

Erythema Infectiosum

Dr. Charles R. Freeble, Jr., Ohio Department of Health, reports an outbreak of approximately 100 cases of erythema infectiosum (also known as Fifth disease) in Cuyahoga County. The cases were first recognized in May; they reached their peak in June and subsided rapidly during July. Dr. A. J. Pearse, Cuyahoga County Health Commissioner, reported that the bulk of the cases were in school-age children with a number of adults and smaller children also evidencing the infection. Multiple cases occurred in some families. The outbreak originated and was fairly well confined to an area of the county where the population has been estimated to include over 25 percent displaced persons recently arrived from central Europe. The outbreak apparently began in this group and soon involved all segments of the population. Early cases were considered to be a mild form of measles or an allergic phenomenon. Several patients experienced a recrudescence of the eruption after apparent recovery. Treatment with antibiotics failed to limit the course of the illness. Apparently no complications or sequelae developed on any of the cases. The absence of posterior cervical lymphadenopathy and normal white differential counts were observed. Later in the course of the outbreak measles began to appear in high incidence, and it was observed that a number of the early cases later developed measles, which inferred a lack of cross immunity. Following the closing of school for the summer, the outbreak gradually subsided. The infection was so mild that many cases were not seen by a physician, and the data presented had to be obtained in retrospect.

Disease of Unknown Etiology

Dr. George W. Cox, Texas State Health Officer, reports an outbreak of illnesses resembling pleurodynia appearing in Fannin County and the nearby area. It is estimated that several thousand cases have occurred. The disease is generally mild, rather self-limited, and of

short duration. Symptoms are frequently severe consisting of fever, 101° to 104°, nausea, vomiting, and particularly pain across the upper abdomen or lower chest. Frequently the patient has complained of difficult or painful breathing. The attack rate is high and the illness has appeared in all age groups. Some of the earlier illnesses were originally thought to be appendicitis because of the localized pain in the lower right quadrant and the sudden onset of fever. Laboratory investigation is proceeding for the isolation of the etiologic agent and includes virus studies of stool and throat washings, and complement fixation and neutralizing tests on paired blood sera. It is surmised at this time there is a probability of Coxsackie infection, possibly of the B type, but proof must await laboratory investigations.

Food Poisoning

Dr. Cox reports an outbreak of food poisoning occurring at Austin, Tex., in a group of missionaries who had two meals at a church. Approximately 30 persons were hospitalized. *Salmonella oranienberg* was found in 48 of 50 stools investigated and in tuna fish, beans, and other food specimens.

Dr. B. N. Diefendorf, New York State Department of Health, has reported a family outbreak of gastroenteritis in Saratoga County, in which canned hamburger was suspected as the vehicle of infection. Although *Staphylococcus aureus* food poisoning was considered probable, laboratory confirmation was lacking.

Comparative Data for Cases of Specified Reportable Diseases: United States

[Numbers after diseases are International List numbers, 1948 revision]

Disease	Total for week ended—		5-year median 1946–50	Seasonal low week	Cumulative total since seasonal low week		5-year median 1945–46 through 1949–50	Cumulative total for calendar year—		5-year median 1946–50
	Sept. 1, 1951	Sept. 2, 1950			1950–51	1949–50		1951	1950	
Anthrax (062)	------	------	------	(1)	(1)	(1)	(1)	46	29	34
Diphtheria (055)	48	82	144	27th	378	545	986	2,386	3,673	5,596
Encephalitis, acute infectious (082)	27	60	28	(1)	(1)	(1)	(1)	687	594	425
Influenza (480–483)	267	294	294	30th	1,332	1,683	1,582	117,387	140,447	130,183
Measles (085)	1,029	701	701	35th	497,612	307,301	586,282	468,911	288,171	551,414
Meningitis, meningococcal (057.0)	51	36	40	37th	3,931	3,630	3,534	2,970	2,717	2,562
Pneumonia (490–493)	427	769	(2)	(1)	(1)	(1)	(1)	46,868	62,131	(2)
Poliomyelitis, acute (080)	1,742	1,625	1,625	11th	12,420	12,349	12,307	13,632	13,480	12,657
Rocky Mountain spotted fever (104)	8	14	16	(1)	(1)	(1)	(1)	273	383	461
Scarlet fever (050) [3]	229	264	290	32d	748	766	973	54,134	40,936	58,324
Smallpox (084)	------	------	------	35th	19	46	71	11	26	50
Tularemia (059)	14	16	15	(1)	(1)	(1)	(1)	469	679	701
Typhoid and paratyphoid fever (040, 041) [4]	98	96	100	11th	1,517	1,821	2,000	1,952	2,331	2,485
Whooping cough (056)	951	1,683	1,683	39th	[5]71,306	111,542	94,320	[5]49,704	90,006	68,302

[1] Not computed.
[2] Data not available.
[3] Including cases reported as streptococcal sore throat.
[4] Including cases reported as salmonellosis.
[5] Addition: Rhode Island, week ended August 18, 4 cases.

Reported Cases of Selected Communicable Diseases: United States, Week Ended September 1, 1951

[Numbers under diseases are International List numbers, 1948 revision]

Area	Diphtheria (055)	Encephalitis, infectious (082)	Influenza (480–483)	Measles (085)	Meningitis, meningococcal (057.0)	Pneumonia (490–493)	Poliomyelitis (080)
United States	**48**	**27**	**267**	**1,029**	**51**	**427**	**1,742**
New England	**2**	**1**	**1**	**132**	**1**	**9**	**53**
Maine				10	1	1	4
New Hampshire							2
Vermont				7			
Massachusetts	2	1		81			13
Rhode Island			1	16			
Connecticut				18		8	34
Middle Atlantic	**9**	**6**		**258**	**9**	**38**	**218**
New York	6	4	(1)	132	4		119
New Jersey	1	2		85	2	12	45
Pennsylvania	2			41	3	26	54
East North Central	**8**	**9**	**11**	**231**	**12**	**72**	**422**
Ohio	5			33	3		74
Indiana	2	2	9	3		4	18
Illinois		4	1	92	6	46	128
Michigan		3	1	49	3	22	98
Wisconsin	1			54			104
West North Central	**1**	**3**	**5**	**34**	**6**	**34**	**240**
Minnesota	1		1			3	35
Iowa				2	1		33
Missouri		1		6	2		64
North Dakota		1	1	14	1	27	5
South Dakota		1		4			14
Nebraska				4			37
Kansas			3	4	2	4	52
South Atlantic	**9**	**1**	**96**	**89**	**8**	**84**	**134**
Delaware							1
Maryland		1	3	43		19	16
District of Columbia				5		3	6
Virginia	1		76	7	2	20	19
West Virginia				7			19
North Carolina	1			2	2		21
South Carolina	7		1	1	1	1	7
Georgia			16	19	2	41	36
Florida				5	1		9
East South Central	**7**	**1**	**1**	**8**	**3**	**16**	**131**
Kentucky			1		3	7	20
Tennessee	3	1		6			56
Alabama	2					5	24
Mississippi	2			2		4	31
West South Central	**8**	**1**	**51**	**50**	**5**	**106**	**157**
Arkansas	1		35	1		9	33
Louisiana					2	4	27
Oklahoma	1		16	3		8	27
Texas	6	1		46	3	85	70
Mountain			**82**	**66**	**2**	**25**	**197**
Montana			4	10		3	11
Idaho				6	1		7
Wyoming							5
Colorado					1	10	90
New Mexico				9		1	9
Arizona			78	34		11	36
Utah				7			39
Nevada							
Pacific	**4**	**5**	**20**	**161**	**5**	**43**	**190**
Washington	2			32			23
Oregon			14	11	1	25	19
California	2	5	6	118	4	18	148
Alaska							4
Hawaii				33		1	

[1] New York City only.

Reported Cases of Selected Communicable Diseases: United States, Week Ended September 1, 1951—Continued

[Numbers under diseases are International List numbers, 1948 revision]

Area	Rocky Mountain spotted fever (104)	Scarlet fever [1] (050)	Smallpox (084)	Tularemia (059)	Typhoid and paratyphoid fever [2] (040, 041)	Whooping cough (056)	Rabies in animals
United States	8	229		14	98	951	100
New England		22			6	74	
Maine		5			3	12	
New Hampshire							
Vermont		2				12	
Massachusetts		8			3	39	
Rhode Island							
Connecticut		7				11	
Middle Atlantic		32		1	9	152	18
New York		21		1	4	76	10
New Jersey		6			1	46	
Pennsylvania		5			4	30	8
East North Central	1	67		3	10	171	6
Ohio		15			1	34	4
Indiana		2			1	14	
Illinois	1	20		2	2	42	
Michigan		21		1	2	45	
Wisconsin		9			4	36	2
West North Central		11		1	5	71	13
Minnesota		1				2	2
Iowa					1	10	5
Missouri		5			3	18	4
North Dakota						4	
South Dakota					1	2	
Nebraska						4	2
Kansas		5		1		31	
South Atlantic	4	25		3	19	122	15
Delaware		1					
Maryland	2	1				7	
District of Columbia		4					
Virginia	2	4				18	5
West Virginia		1			8	40	3
North Carolina		13			3	21	
South Carolina				2	3		3
Georgia		1		1	5	24	4
Florida						12	
East South Central	2	26		1	14	51	28
Kentucky		5			5	18	18
Tennessee	1	13			7	19	3
Alabama	1	5				11	4
Mississippi		3		1	2	3	3
West South Central		6		2	11	194	19
Arkansas		1		1	1	10	4
Louisiana					2		
Oklahoma		2			2	10	2
Texas		3		1	6	174	13
Mountain	1	7		1	4	41	
Montana		1				1	
Idaho						3	
Wyoming				1			
Colorado		1			2	19	
New Mexico					2	7	
Arizona		1				10	
Utah	1	4				1	
Nevada							
Pacific		33		2	20	75	1
Washington		1				3	
Oregon		3			1	6	
California		29		2	19	66	1
Alaska							
Hawaii							

[1] Including cases reported as streptococcal sore throat.

[2] Including cases reported as salmonellosis.

FOREIGN REPORTS

CANADA

Reported Cases of Certain Diseases—Week Ended August 18, 1951

Disease	Total	New found-land	Prince Ed-ward Island	Nova Scotia	New Bruns-wick	Que-bec	On-tario	Mani-toba	Sas-katch-ewan	Al-berta	Brit-ish Co-lum-bia
Brucellosis	6					5	1				
Chickenpox	172	2		6		17	81	9	18	30	9
Diphtheria	2					2					
Dysentery, bacillary	14					9		2			3
Encephalitis, infectious	1								1		
German measles	99			7		13	37	2	17	13	10
Influenza	22			17			2				3
Measles	339	2		40		124	37	10	23	70	33
Meningitis, meningo-coccal	1						1				
Mumps	116	2		2		26	34	10	20	9	13
Poliomyelitis	170	2		10		14	125		8	8	3
Scarlet fever	78	2			1	25	8	12	7	7	16
Tuberculosis (all forms)	210	30		1	2	94	26	24	11	11	11
Typhoid and para-typhoid fever	20	1				15	2				2
Venereal diseases:											
Gonorrhea	250	1		10		61	51	16	25	32	54
Syphilis	60			4	5	25	18	2		3	3
Primary	7					2	3			2	
Secondary	1						1				
Other	52			4	5	23	14	2		1	3
Other forms	1					1					
Whooping cough	157			1	2	51	52	7	9	8	27

CUBA

Reported Cases of Certain Diseases—4 Weeks Ended July 28, 1951

Disease	Total	Pinar del Rio	Habana		Matan-zas	Santa Clara	Cama-guey	Oriente
			Habana City	Total				
Cancer	64	2		10	12	24		16
Chickenpox	3		2	2				1
Diphtheria	7		5	5	2			
Leprosy	6			5				1
Malaria	16		1	1				15
Measles	35		15	15			14	6
Tuberculosis	67			5	10	22	8	22
Typhoid fever	40	3	2	3	1	12	5	16

MADAGASCAR

Reported Cases of Certain Diseases and Deaths

June 1951

Disease	Aliens		Natives	
	Cases	Deaths	Cases	Deaths
Beriberi			1	1
Bilharziasis	1		6	
Dysentery:				
Amebic			22	
Bacillary	2		6	
Erysipelas			2	
Influenza	36		5,115	7
Leprosy	1		13	
Malaria	139	1	19,484	60
Measles	33		200	
Mumps			89	
Plague			1	
Pneumonia (all forms)	2		555	52
Puerperal infection			2	
Tuberculosis, respiratory	2		66	9
Typhoid fever	1		8	1
Whooping cough	7		190	7

July 1951

Disease	Aliens		Natives	
	Cases	Deaths	Cases	Deaths
Beriberi			1	1
Bilharziasis	1		17	
Diphtheria	2	1	3	
Dysentery, amebic	4		76	3
Erysipelas			3	
Influenza	6		4,821	18
Leprosy	1	1	18	
Malaria	99		26,847	65
Measles	34		76	
Meningitis, meningococcal	1		1	
Mumps			46	
Plague			6	6
Pneumonia (all forms)			639	70
Puerperal infection			7	
Trachoma			1	
Tuberculosis, respiratory	3		88	10
Typhoid fever	3		4	
Whooping cough	1		194	3

Note.—No report was received from Madagascar for May 1951.

REPORTS OF CHOLERA, PLAGUE, SMALLPOX, TYPHUS FEVER, AND YELLOW FEVER RECEIVED DURING THE CURRENT WEEK

The following reports include only items of unusual incidence or of special interest and the occurrence of these diseases, except yellow fever, in localities which had not recently reported cases. All reports of yellow fever are published currently.

Cholera

Burma. A total of 12 cases of cholera was reported in Tavoy for August 12–18. These are the first cases reported.

India (French). A total of 66 cases and 33 deaths from cholera was reported for July 21–31 for French India. Included in the total are 8 cases and 4 deaths in Pondicherry, 57 cases and 29 deaths in

Pondicherry dependencies, and 1 first case in Yanaon. For the period August 1–10, 20 cases were reported in Pondicherry.

Plague

India. In Mysore, for the period June 29–August 4, a total of 58 cases and 14 deaths was reported. For August 5–11, a total of 43 cases and 21 deaths was reported.

Indochina. For the period August 5–11, a total of two cases of plague was reported in Phanthiet.

Madagascar. For the period August 1–10, a total of three cases and three deaths was reported; and for the period August 11–20, a total of two cases and one death was reported.

Smallpox

Cameroons (French). For the period August 1–10, a total of 14 cases was reported. Included in the total were 7 cases in Benove, and 5 in Marguiwandala.

French West Africa. For the period August 1–10, a total of 162 cases and 31 deaths from smallpox was reported. The total included 13 cases previously reported for the same period from Upper Volta. For the period August 11–20, 9 cases and 1 death were reported from Dahomey.

Gold Coast. For July 2, in the Wa district, a total of one case and one death was reported.

Typhus Fever

Egypt. For the period August 5–11, one case of typhus fever was reported in Cairo.

Iran. For the period July 14–20, preliminary reports give one case and one death from typhus fever in Ahvaz. For the period July 21–27, preliminary reports of typhus fever give one case in Malayer and one case in Tauriz.

New Caledonia. For the period July 1–10, one case of typhus fever was reported.

Tunisia. For the period July 11–20, one case of typhus fever was reported in Beja. And for the period July 21–31, three cases of typhus were reported in Gafsa.