Preparation of Highly Purified Concentrates of *Coxiella burneti*[1] (35125)

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(Introduced by J. L. DeMeio)
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Various methods for the concentration and purification of *Coxiella burneti* have been described. These include the use of diethyl ether (1), Celite (2), centrifugation from 1 *M* KCl followed by treatment with diethyl ether (3), treatment with dextran sulfate followed by centrifugation (4), density gradients (5), and cellulose anion exchangers (6).

Of these methods, perhaps those utilizing diethyl ether are most commonly employed for the purification of formalin-inactivated *C. burneti*. Diethyl ether is used in these procedures to promote the separation of the organism from the tissue in which it has been propagated. Although the solvent has certain useful properties which include its bacteriostatic action and its lipid dissolving ability, it is also a highly inflammable solvent and as such does not readily lend itself to any large scale procedures.

Recent studies in these laboratories have been directed toward the concentration and purification of *C. burneti* without the use of diethyl ether and it is the purpose of this communication to describe a relatively simple method of preparing highly purified concentrates without the application of hazardous solvents.

Materials and Methods. A fourth egg passage of the Henzerling strain of *C. burneti* was obtained as a frozen 20% yolk sac suspension from the U.S. Army Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland.

A fifth egg passage was prepared in these laboratories and passed once in guinea pigs followed by three passages in eggs. The third egg passage was stored as a 20% yolk sac suspension at −60° to provide seed material. All eggs used in these studies were leukosis-free chicken eggs (SPAFAS).

Seven-day-old embryonate eggs were inoculated with a dilution of seed material predetermined to give a 20% mortality between days 7 and 8 postinoculation. Yolk sacs were harvested from the surviving eggs on the day showing a 20% mortality, weighed and stored at −60°.

Frozen yolk sacs were partially thawed and suspended in sufficient cold (4°) phosphate buffered saline (0.15 *M* NaCl, 0.006 *M* Na_2HPO_4, pH 7.0) to make a 20% (w/v) suspension. An aerosol-free Waring Blendor was used for this purpose employing a suspending time of 3 min.

The 20% yolk sac suspension was diluted with an equal volume of 2 *M* NaCl containing 1% (v/v) formalin (Fisher) and stirred at 24° for 20 hr. The suspension was transferred to a fresh container and stirred for 40 hr at 4°.

The inactivated 10% yolk sac suspension was centrifuged for 2 hr at 11,400*g* at 4°. The lipid pellicle was dislodged, drawn off along with the supernatant liquid and discarded. The yellow pellet was suspended in sufficient 1 *M* NaCl containing 0.5% (v/v) formalin to give a final volume equivalent to one-tenth the volume of the original 20% yolk sac suspension.

Freon treatment. One volume of the resuspended pellet at 4° was adjusted to pH 5.7 with 2 *N* HCl and cooled to 0–2° in an ice bath. Four-tenths of a volume of cold (0–2°) absolute ethanol was added slowly with stirring. Stirring was continued for 30 min, and

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1 vol of cold (0–2°) trichlorotrifluoroethane (Freon 113, Dupont) was added. The mixture was stirred for an additional 30 min. The mixture was transferred to a graduated cylinder and permitted to stand at 4° for 18 hr. The upper milky-white first water phase was carefully drawn off, adjusted to pH 6.8 with 1 *N* NaOH and stored at 4°. The residual denatured protein and lipid-containing Freon phases were re-extracted by stirring for 30 min at 4° with 1 vol of a special water phase previously prepared by equilibrating at 4° a mixture of 1 vol of 1 *M* NaCl containing 0.5% formalin (v/v) and 0.4 vol of absolute alcohol with 1 vol of Freon 113. The second extraction was also permitted to stand for 18 hr at 4° and the upper second water phase was removed as before. The second water phase was adjusted to pH 6.8 with 1 *N* NaOH, pooled with the first water phase and stored at 4°.

Brushite treatment. Brushite (7) which had previously been equilibrated with 1 *M* NaCl in 0.001 *M* sodium phosphate buffer, pH 7.0, was used to further purify the pooled water phase fraction. Columns measuring 50 mm in diameter by 120 mm in length were found satisfactory for processing 150- to 200-ml aliquots of pooled water phase. Column packing pressure was found to be critical and could not exceed 1 psi or high losses would be encountered. The pooled water phase fraction was followed by the same solution used to equilibrate the brushite. Flow rates were 0.5 to 1.0 ml/min and collection of effluent was initiated at the first sign of turbidity and terminated when turbidity dropped sharply.

The brushite effluent was centrifuged for 2 hr at 11,400*g* at 4° and the water-clear supernatant liquid was discarded. The sedimented white pellet was suspended in sufficient cold (4°) phosphate buffered saline to obtain a final volume equivalent to one-half that of the effluent centrifuged. The suspended pellet was stirred vigorously for 15 min at 4° and clarified by centrifugation for 10 min at 280*g* at 4°.

Assay procedures. Optical density measurements at 420 mμ were made in phosphate buffered saline using the Spectronic 20 Colorimeter (Bausch and Lomb). Total nitrogen determinations were made by the usual micro-Kjeldahl digestion technique, followed by Nesslerization and absorption measurements at 440 mμ. Total solids were obtained by dialyzing samples exhaustively against distilled water at 4° and drying aliquots of the dialyzed preparation at 95° until a constant weight was realized. Complement fixation assays were performed according to the microtiter version of The Laboratory Branch of The Communicable Disease Center test (8). Phase characterization was accomplished by the complement fixation test in which early (17) day and late (45) convalescent guinea pig sera were used. Egg protein amounts were estimated by the use of the complement fixation test using a guinea pig antiserum prepared against egg albumin and yolk sac by the method of Lackman (9). As little as 0.1 to 0.5 μg/ml of egg albumin could be detected by this procedure.

Results. For the comparision of the degree of purification and yield obtained at each step, samples were removed and analyses of total nitrogen, optical density, and complement fixation content were obtained. The initial centrifugation step removed a considerable amount (87%) of nonrickettsial nitrogen (Table I). Eleven percent of the nitrogen of the high speed pellet was removed by treatment with Freon 113 leaving the pooled Freon water phase with a high concentration of rickettsiae contaminated with small amounts of tissue debris, yellow pigment, and nonsedimentable egg antigen. The tissus debris and yellow pigment were removed by the brushite treatment and the egg antigen was reduced to undetectable levels by the second sedimentation.

Within the limits of the complement fixation assay, overall yields were 59%.

The final produce was a white, turbid suspension which, when examined microscopically as a stained smear (10), appeared to consist wholly of rickettsia-like bodies. The final product showed a marked reaction in the complement fixation test when late (45 day) convalescent guinea pig serum was used, but gave negative results when tested with early (17 day) convalescent sera or with an anti-

TABLE I. Purification of Formalin-Inactivated *C. burneti*; Henzerling Strain, Fourth Egg Passage.

Fraction	Vol (ml)	Nitrogen (μg/ml)	OD @ 420 mμ	Complement-fixing titer[a]		
				Phase 1	Phase 2	Egg[b]
20% yolk sac suspension	670	2300	96	64		
10% yolk sac suspension	1340	1060	34	32		
High speed supernatant no. 1	1340	920	28	AC @ undil.		
200% yolk sac pellet	67	800	27.5	512		
First Freon water phase	71.5	552	10.4	256	8	
Second Freon water phase	70.5	106	1.7	32	AC @ 2	
Pooled water phases	142.0	336	6.8	128	4, AC @ undil.	
Brushite effluent	198.0	144	3.2	64	Neg	
High speed supernatant no. 2	195.0	11.5	0.01	4		
Resuspended pellet	99.0	288.0	6.4	256	Neg	Neg
Clarification residue	9.9		0.2	16	Undil.	
Purified antigen	99.0	264	6.3	256	Neg	Neg

[a] Reciprocal value.

[b] One CF unit equivalent to 0.1 to 0.5 μg of egg albumin.

egg guinea pig serum capable of detecting 0.1 to 0.5 μg/ml of egg albumin. The average nitrogen content of the final product on a dry weight basis was 11.7%.

The relationship between optical density (turbidity) and *C. burneti* concentration was investigated. Samples of a purified rickettsial concentrate were dialyzed exhaustively against distilled water at 4° and dried to constant weight at 90°. Four samples of the concentrate prior to dialysis were appropriately diluted with buffer and the optical density of the dilutions was measured at 420 mμ. The results agree well with those reported elsewhere (11).

Summary. Procedures for the preparation of highly purified concentrates of formalin-inactivated *Coxiella burneti* were developed. These procedures include the use of centrifugation, fluorocarbon, and brushite treatments. The final product was free of detectable host (egg) antigen and had only phase 1 complement-fixing activity. The relationship between concentration and optical density was confirmed for the concentrated material.

1. Craigie, J., Can. J. Res. **23**, 104 (1945).

2. Shepard, C. C., and Topping, N. H., J. Immunol. **55**, 97 (1947).

3. Ormsbee, R. A., J. Immunol. **88**, 100 (1962).

4. Davis, J. L., and Patrick III, W. C., Appl. Microbiol. **13**, 99 (1965).

5. Ribi, E., and Hoyer, B. H., J. Immunol. **85**, 314 (1960).

6. Hoyer, B. H., Bolton, E. T., Ormsbee, R. A., LeBouvier, G., Ritter, D. B., and Larson, C. L., Science **127**, 859 (1958).

7. Tiselius, A., Hjerten, S., and Levin, O., Arch. Biochem. Biophys. **65**, 132 (1956).

8. U. S. Pub. Health Serv. Pub. Health Monogr. **74**, (1965).

9. Lackman, D. B., Amer. J. Pub. Health Nat. Health **52**, 92 (1962).

10. Gimenez, D. F., Stain Technol. **39**, 135 (1964).

11. Fiset, P., Ormsbee, R. A., Silberman, R., Peacock, M., and Spielman, S. H., Acta Virol. **13**, 61 (1969).

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