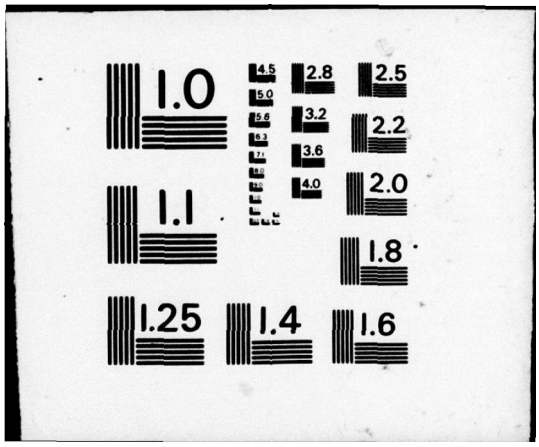
AD-A036 017 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/15
PURIFICATION TECHNIQUES FOR LIVING RICKETTSIAE,(U)
FEB 77 M CAPPONI, J GIUNTINI, K KAWAI

UNCLASSIFIED USAMRIID-MUL-0476 NL

1 OF 1
ADA036017

END
DATE FILMED
3-77


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

AD

12
B.S.

TRANSLATION NO.: MUL 0476

TITLE: Purification techniques for living rickettsiae

AUTHOR(S): Capponi, M., J. Giuntini, and K. Kawai

REFERENCE: Ann. Inst. Pasteur 121:43-48, 1971

DISTRIBUTION STATEMENT

**Approved for public release;
distribution unlimited**

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

Fort Detrick, Frederick, Maryland 21701

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Purification techniques for living rickettsiae		5. TYPE OF REPORT & PERIOD COVERED Translation
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(a) M. Capponi, J. Giuntini, and K. Kawai		8. CONTRACT OR GRANT NUMBER(a) USAMRIID MUL-0476
9. PERFORMING ORGANIZATION NAME AND ADDRESS Ann. Inst. Pasteur 121:43-48, 1971		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS USAMRIID LIBRARY, Fort Detrick, Frederick Md.		12. REPORT DATE 22 Feb 77
		13. NUMBER OF PAGES 9
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release: distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Rickettsiae
Purification techniques

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

US Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Md. 21701

AD _____

PURIFICATION TECHNIQUES FOR LIVING RICKETTSIAE

M. CAPPONI, J. GIUNTINI, and K. KAWAI

ANN. INST. PASTEUR 121:43-48, 1971

Translated by Phebe W. Summers

SUMMARY

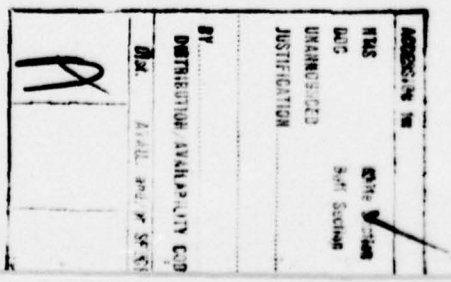
TECHNIQUES FOR THE PURIFICATION OF LIVING RICKETTSIAE

Numerous techniques for the purification of Rickettsiae exist at the present moment. Their high number indicates the extent of the problem. To obtain purified Rickettsiae which are still viable, techniques which involve simple centrifugation in the presence of KCl M/1 or, even better, those in which either Amberlit or Sephadex is added to the centrifuged preparation, are recommended. These methods are only slighty more complicated than the technique of Craigie and have the advantage of yielding living suspensions in which the bacterial coat is only slightly altered and whose virulence is only slightly reduced.

The problem of purification of rickettsiae can no longer be posed for R. quintana which has been cultivated on enriched agar without cells (9), in which case one suspends colonies taken from an agar base.

While it can be carried out on a homogenate of lungs of mice inoculated by the intranasal route, lungs of rabbits inoculated by the intratracheal route, or even more easily on yolk sac membranes, the problem is more complex. One must separate the cells and tissue debris; this is not feasible by physical or chemical procedures, which generally damage the rickettsial walls.

Various techniques have been utilized since Castaneda (11), that of Craigie with ether, among others. Other ingenious procedures have been successively used (2-5): density gradients and use of sucrose and of 1 M KCl (Ribi and Hayer); dextran and $CaCl_2$ (David and Patrick; K tartrate (Allen); celite (Shepard); ion exchange resins such as Amberlite; or DEAE-cellulose (Fiset and Silbermann), etc. After several



purification attempts with trypsin and polymyxin, fluorocarbon (? Genetron) and by 1 M KCl, we have twice tried a purification using Whatman AE-81 paper, then Amberlite and finally Sephadex G-50. The problem of obtaining living rickettsiae has evidently been resolved by this second series of techniques.

Materials and Methods

In all cases the yolk sacs have been homogenized in an Ultra-Turrax grinder for 1 min. Only cultures of nonpathogenic strain E of R. prowazeki incubated in eggs have been used.

Trypsin and polymyxin B have been utilized according to the method of Woolridge and Grayston (10) as applied to the purification of trachoma elementary bodies. Ground membranes of infected incubated eggs were suspended in Sorensen buffer, adjusted to pH 8 with 1% carbonate, then treated with trypsin at a concentration of 0.2% and placed in a 37 C water bath for 3 hr. After a new addition of trypsin (0.1-0.2 ml/100 ml), 2 centrifugations were carried out, 100 and then 1800 rpm. The supernatant was then centrifuged at 10,000 rpm 2 times and the pellet taken up in buffer. Polymyxin B was added at a rate of 80 mg/ml; the whole was cooled to 4 C. A precipitate formed; light centrifugation permitted recovery of the supernatant, rich in rickettsiae.

The Genetron was used according to the technique for elementary bodies of trachoma developed by Bernkoff and Orfila (4). Forane 113, produced at little cost, is stocked in 2-liter containers. Mixing several yolk sacs, rich in R. prowazeki strain E, with 0.85% NaCl and Genetron,

in a proportion of 40 ml of membranes to 200 ml of physiologic water and 66 ml of Genetron, one homogenizes and centrifuges at 1800 rpm for 5 min. After removing the superficial lipid pellicle one decants (1st supernatant); 200 ml of physiologic water to obtain the 2nd supernatant. Combining these supernatants and recommencing the whole operation, one obtains after quick centrifugation a pellet very rich in rickettsiae, which can be suspended as desired in physiologic water.

1 M KCl has been used in the combination for centrifugation, according to the techniques of Ribi and Hayer (5). Frozen yolk sacs collected in distilled water were thawed, mixed with molar KCl in a proportion of 40 ml of 1 M KCl to 8 gm of yolk sac in distilled water. After centrifugations at 1500 rpm for 10 min and recovery of the supernatant, this was centrifuged 15 min at 9000 rpm and the pellet was taken up in 1 M KCl. Finally, 2 centrifugations at 9000 rpm yielded a pellet which was suspended in a small quantity of distilled water.

Whatman Paper. We have used sterilized diamino-ethyl-cellulose AE-81, a weak anion exchanger (5.0 mEq/m^2 capacity, diffusion speed 15 min).

This paper, placed on a sterile funnel, is treated with an acidifying solution, then again with a pH 8 solution; the filtrate is the semipurified suspension. After 3 centrifugations of the yolk sac in physiologic saline, 2 slow and one fast, the pellet is taken up in a small quantity of physiologic water and filtered on the cellulose-treated paper. An opalescent almost pure suspension, and rich in rickettsiae (strain E of R. prowazeki), has been obtained at a pH slightly less than 7.

Amberlite Treatment. The technique follows that of Yamamoto and Kawamura (8). The Amberlite used was the resin LRC-50H RP, sterilized at 110 C by autoclaving after treatment with an alkaline then an acidic solution. To 30 ml of the rickettsial suspension of the same strain, 10% sterilized resin was added with 30 ml of Bovarnick-Snyder (SPG). A first centrifugation for 10 min at 1000 rpm is followed by a second at 9000 rpm for 25 min after taking up the intermediate phase. The pellet mixed again in 15 ml of Bovarnick solution is centrifuged at 1000 rpm. The supernatant is removed and recentrifuged at 9000 rpm for 25 min. This pellet in Bovarnick solution contains pure rickettsiae; storage is at -30 C.

Sephadex G-50 Technique has not been described elsewhere and seemed interesting to compare with the Amberlite method; the materials used and the methods were similar to the preceding. Sephadex G-50 replaces the Amberlite and the last pellet is taken up in Bovarnick solution and stored at -30 C.

A second technique is offered for Sephadex column chromatography. After grinding 1 min in the Ultra-Turrax, 10 yolk sacs rich in rickettsiae (strain E), 3 centrifugations are carried out: 2 slow ones at 1400 rpm, with recovery of the intermediate strain, and a rapid 3rd one at 9000 rpm, with recovery of the pellet in 30 ml of physiologic water. A Sephadex G-50 column with 10 gm/150 ml saline buffer at pH 7.2 was used. The cellulose powder was previously sterilized at 110 C by autoclaving. The

rickettsial suspension is poured into the upper part of the sterile column (the lower part has been plugged with sterile cotton); after 2 hr a rickettsial suspension was recovered and placed at -30 C.

For electron microscopy, after centrifugation, the suspensions were prepared in distilled water and placed on grids for examination. The suspensions dried with UV light were shadowed with an alloy of gold and platinum at an angle of 15°. The examinations made with a Siemens EM were carried out with a 80 Kv electron beam, at an enlargement of 8500. The final magnification obtained photographically was 18,000.

Results

Here briefly are the results obtained and presented in the 2 tables.

I. Inoculation of 7-day incubated eggs by the yolk sac route and numbers of eggs containing rickettsiae (Table I).

Table I

Techniques	No. eggs	No. eggs containing rickettsiae	Embryos dying before the 10th day
Trypsin and polymyxin	10	0	0
Fluorocarbon (Genetron)	10	0	0
KCl (1 M)	10	2	2
Whatman paper	20	10	0
Amberlite	10	5	5
Sephadex	20	6	0

It can be seen that with the trypsin-polymyxin and Genetron methods, no rickettsiae survive, as anticipated. In contrast with 1 M KCl, contrary to the opinion of other authors, some rickettsiae are still intact and in 20% of the eggs one sees dead embryos before the 10th day, which assumes fairly good virulence. With Whatman paper half the eggs survive, but virulence is less: no embryo died before the 10th day. It is the same with Sephadex: from one-third of the eggs, one recovers living rickettsiae, but the embryos die late. On the other hand, with Amberlite, half the eggs survive and the embryos die before the 10th day. Nevertheless, it should be noted that with this process one can see some contaminants because the manipulations are longer than with Sephadex.

II. Microscopic Purity of the Suspensions.

To the naked eye, the suspensions always have the same clear opalescent appearance. But if one rates the rickettsial purity by light and electron microscopy from $\pm$ to 1+, 2+, 3+ and 4+ the suspension is imperfectly purified as shown in Table II.

Table II

	Light Microscopy	EM
Trypsin and polymyxin	2+	ND
Fluorocarbon (Genetron)	3+	ND
1 M KCl	2+	ND
Whatman paper	1+	$\pm$
Amberlite	2+	+
Sephadex	2+	+

The purity of the suspension viewed by light microscopy is, therefore, best with Genetron; it is good with Amberlite or Sephadex; it is a little less with simple filtration on Whatman paper. By EM with Amberlite or Sephadex (Fig. 1), one obtains a fairly good suspension; it is less good with Whatman paper.

FIG. 1. — *Rickettsia prowazeki* souche E après purification par le Sephadex. Grossissement 18 000 (microscope Siemens).

Discussion

Obviously, one can for such a problem of purification, be satisfied with repeated centrifugations; but it is difficult to obtain in this manner rickettsiae of much purity.

When one obtains a rickettsial suspension after purification with Amberlite or Sephadex, one can preserve them in sealed ampules at -30 C.

They store better at -70 C, under dry ice. Stock can be stored for several weeks, even several months. There may be some loss of virulence.

Treatment by resins seems to be more logical than treatment by inert powder such as Sephadex. Nevertheless, the manipulations with the Amberlite technique are long and subject to contamination. It seems that the Sephadex technique which uses sterilizable cellulose particles, without previous preparation is simplest and preferable. It should be noted that a portion of the rickettsiae is not living.

Treatment with 1 M KCl perhaps shortens the procedure, without damage to the rickettsiae. It is a simple technique, recommended in certain cases, because it is rapid and carried out with ease.

As for the other treatments for rickettsial suspensions, such as those which utilize trypsin and polymyxin or treatment by Genetron, they are very tempting, but they have the major inconvenience of decreasing the numbers of rickettsiae and totally inactivating them.

Finally, to the techniques described, one can always add centrifugations: the only inconvenience of their repetition is perhaps the lengthy procedure, decreasing the number of rickettsiae or the risk of introducing contaminants.

BIBLIOGRAPHIE

[1] DAVIS (R. L.) and GRAYSTON (J. Th.). Further studies with a complement fixation test for trachoma. *Ann. N. Y. Acad. Sci.*, 1962, *98*, 314-328.

[2] FISET (P.) et SILBERMAN (R.). Purification des rickettsies au moyen d'échangeurs d'ions. *Arch. Inst. Pasteur Tunis*, 1966, *43*, 231.

[3] KATZENELSON (E.) and BERNKOPF (H.). Serologic differentiation of trachoma strains and other agents of the psittacosis-lymphogranuloma venereum-trachoma group with the aid of the direct fluorescent antibody method. *J. Immunol.*, 1965, *94*, 467.

[4] LÉPINE (P.). Techniques de laboratoire en Virologie humaine. Masson édit., Paris, 1964.

[5] RIBI (E.) and HOYER (B. H.). Purification of Q fever rickettsiae by density-gradient sedimentation. *J. Immunol.*, 1960, *85*, 314.

[6] SHEPARD (Ch. C.) and TOPPING (N. H.). Preparation of suspension of rickettsiae from infected yolk sac without the use of ether. *J. Immunol.*, 1967, *55*, 97.

[7] SILBERMAN (R.) and FISET (P.). Method for counting Rickettsiae and Chlamydia in purified suspensions. *J. Bact.*, 1968, *95*, 259.

[8] TAMIYA (T.). Recent advances in studies of tsutsugamushi disease in Japan. Medical Culture Inc. Tokyo, 1962.

[9] VINSON (J. W.) and FULLER (H. S.). Studies on trench fever. Propagation of rickettsia-like organisms from a patient's blood. *Path. Microbiol.*, 1961, *24*, 152.

[10] WOOLRIDGE (R. L.) and GRAYSTON (J. T.). Further studies with a complement fixation test for trachoma. *Ann. N. Y. Acad. Sci.*, 1962, *98*, 314.

[11] ZDRODOVSKII (P. F.) and GOLINEVICH (E. H.). The Rickettsial Diseases. Pergamon Press, 1960.