SAFETY TEST FOR Q FEVER VACCINE

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Viable *Coxiella burnetii* are usually demonstrated in the laboratory by infecting either guinea pigs or embryonated eggs. Guinea pig infection is manifested by a febrile illness and the development of specific complement-fixing antibodies after intraperitoneal injection of the fluid under examination (Smadel et al., 1948). When embryonated eggs are used, the material is injected into the yolk sac and infection is confirmed by demonstrating rickettsiae in impression smears of the yolk sac membranes; a negative test is reported when such smears are still negative after two blind passages of pooled yolk sacs from surviving eggs (Ransom and Huebner, 1951; Malloch and Stoker, 1952; Sokolova and Fedorova, 1958).

Administration of a formalin-inactivated Q fever vaccine to man and guinea pigs after exposure to a low infecting dose of *C. burnetii* may prevent the development of clinical disease. Considering the elimination of the febrile disease, together with the fact that a potent vaccine elicits a good antibody titer in guinea pigs, the sensitivity of the guinea pig test in detecting persistence of viable rickettsiae in Q fever vaccine becomes questionable. The studies reported here were undertaken to determine the relative sensitivity and reliability of the two methods for detecting viable organisms in a vaccine.

MATERIALS AND METHODS

Strain. The 22nd egg passage of the Henzerling strain of *C. burnetii* was received as a 20 per cent yolk sac suspension from Miss E. Jackson, National Institutes of Health.

Preparation of vaccine. Q fever vaccine was prepared by the method described by Smadel et al. (1948) with the exception that the 7-day-old embryonated eggs were inoculated with 0.2 ml of a 10^{-3} dilution of the seed preparation and yolk sacs were harvested on the 8th day following inoculation.

Preparation of a concentrate of vaccine. Formalin inactivated Q fever vaccine was centrifuged at 4

C for 2 hr at 22,620 $\times$ G. The supernatant fluid was discarded and the sediment resuspended to $\frac{1}{10}$ the original volume with physiological saline containing 500 units of penicillin per ml. This procedure eliminated formalin and merthiolate.

Eggs. Embryonated eggs (7-day-old) from White Leghorn hens raised on antibiotic-free feed were used. The yolk sacs were inoculated with 0.5 ml of the various test suspensions. The eggs were incubated at 36 C for a maximum of 12 days.

Guinea pigs. Hartley strain guinea pigs, ranging in weight from 275 to 375 g, were obtained from Fort Detrick, Maryland. Guinea pigs of this strain from this source had been found to respond to measured doses of *C. burnetii* with a predictable febrile course. The various test suspensions were inoculated intraperitoneally in 1.0 ml amounts. Rectal temperatures were taken for 21 days, at the end of which time the animals were bled and complement-fixation determinations performed on the sera. Temperatures of 103.8 F for 2 or more days were considered to be indicative of infection.

Serological tests. Complement-fixation tests were performed using the method described by Smadel (1956), using overnight fixation and 4 units of commercial antigen prepared from the Nine Mile strain.

RESULTS

Effect of concentration of vaccine on growth of C. burnetii in embryonated eggs. To assure that a 10 $\times$ concentrate would not be toxic to eggs and to determine if minimal numbers of live rickettsiae could be detected in embryonated eggs in the presence of a 10 $\times$ concentration of vaccine, two serial 10-fold dilutions of a seed suspension were made, one in saline containing 500 units of penicillin per ml (Jackson, 1951) and the other in the 10 $\times$ concentrate. One-half ml of the 10^{-7} through 10^{-10} dilutions of both was inoculated into eggs

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via the yolk sac route. Eggs inoculated with only the 10× concentrate served as controls.

As seen in table 1, few deaths occurred in the 1st egg passage and no visible rickettsiae could be detected by microscopic examination of stained smears of yolk sacs from live or dead embryos. In the 2nd egg passage of yolk sac pools of each of the individual dilutions almost all embryos died and without exception this corresponded with a positive microscopic identification of rickettsiae. Few deaths occurred in the two passages of the control group and yolk sacs from these eggs were negative for rickettsiae.

Comparative sensitivity of embryonated eggs and guinea pigs to minimal numbers of rickettsiae in vaccine. A sample of a 10 per cent Q fever vaccine was centrifuged at 4 C for 2 hr at 22,620 × G. The supernatant was discarded and the sediment resuspended in the saline-penicillin diluent to a 5 per cent suspension, the proposed final concentration of the vaccine. This procedure eliminated formalin and merthiolate. Ten-fold serial dilutions of a Henzerling seed suspension were then made in the resuspended vaccine and in saline-penicillin solution and 0.5 ml of the 10^{-7} through 10^{-11} dilutions was inoculated into eggs, whereas 1 ml of the 10^{-7} through 10^{-10} dilutions was inoculated intraperitoneally into guinea pigs. The elevated temperatures of the guinea pigs and complement-

fixation results are listed in table 2, and the results of the egg passages are shown in table 3.

The guinea pigs inoculated with rickettsiae diluted in saline showed the expected relationship between number of organisms, temperature, and complement-fixation response in that the time of fever onset was delayed with dilution and the complement-fixation responses occurred in only those animals showing fever. These findings in guinea pigs were similar to those of Robbins *et al.* (1946) in that little clinical evidence of disease other than fever occurred in guinea pigs with injection of small doses of infectious material and it was not uncommon for some animals to show no temperature response to the injection of these materials. None of the uninoculated controls, housed with the infected animals, showed either fever or a positive complement-fixation titer. In the animals inoculated with rickettsiae diluted in vaccine the time of fever onset was again delayed with dilution; in addition, approximately half as many of the animals developed fevers in this group (both in total and with each dilution) as in the group which received rickettsiae diluted in saline. In most cases, the addition of live rickettsiae to the vaccine appeared to result in a higher complement-fixation than was obtained with the vaccine alone.

In the 1st egg passage of *C. burnetii* diluted in either saline or vaccine, no visible rickettsiae were detected in the yolk sacs. In the 2nd egg passage, almost all eggs died in saline dilutions ranging from 10^{-7} through 10^{-11} and in vaccine dilutions from 10^{-7} through 10^{-10}. This correlated with microscopic identification of rickettsiae in the yolk sacs. The eggs inoculated with the 1st egg passage pool of the 10^{-11} dilution in vaccine were negative for visible organisms, but after the 3rd egg passage all embryos died and their yolk sacs were positive for rickettsiae. All controls (vaccine and uninoculated) were negative.

TABLE 1

Effect of a ten-fold concentration of Q fever vaccine on the growth of Coxiella burnetii in embryonated eggs

Dilution of Infected Yolk Sac	Diluent							
	10× Vaccine				Saline-penicillin			
	1st passage		2nd passage		1st passage		2nd passage	
	Deaths*	Smear	Deaths	Smear	Deaths	Smear	Deaths	Smear
10^{-7}	1/8	—	9/9	+	0/9	—	9/9	+
10^{-8}	0/8	—	9/9	+	0/9	—	9/9	+
10^{-9}	3/10	—	8/8	+	1/8	—	8/8	+
10^{-10}	0/8	—	7/9	+	0/9	—	8/8	+
None	2/24	—	1/8	—				

* (Number of embryos found dead between the 5th and 12th postinoculation days of incubation)/(Total number of embryos alive on the 5th postinoculation day).

DISCUSSION

Although both guinea pigs and embryonated eggs appeared to be about equally capable of measurably responding to small numbers of live rickettsiae in saline, this was not true with live rickettsiae in vaccine. Neither the vaccine nor the 10-fold concentrated vaccine interfered with the growth of the organism in the embryonated egg and in 3 passages of the 10^{-11} dilution of live rickettsiae in vaccine, the number of *C. burnetii*

TABLE 2

Fever response and complement-fixing antibodies in guinea pigs inoculated with Henzerling strain of Coxiella burnetii diluted in saline and in Q fever vaccine

Saline

Dilution	Guinea Pig No.	Fever response at day postinoculation* 4	5	6	7	8	9	10	11	12	13	14	Complement Fixation Titer at 21 Days
10^{-7}	1		×					×	×	×			1000
	2		×	×	×	×	×						2000
	3			×	×	×	×						1000
	4			×	×	×	×						2000
	5			×	×	×	×						2000
10^{-8}	1		×	×	×	×	×						2000
	2				×	×	×						2000
	3							×					<1
	4				×	×	×						2000
	5						×						<1
10^{-9}	1					×	×	×					<1
	2					×	×	×	×				1000
	3					×	×	×	×				1000
	4						×	×					500
	5						×						500
10^{-10}	1				×								<1
	2							×	×	×			500
	3							×	×	×			500
	4									×			<1
	5									×	×		100

Vaccine

Guinea Pig No.	Fever response at day postinoculation* 4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Complement Fixation Titer at 21 Days
1																			8
2																			2000
3						×													1000
4					×	×													2000
5																			2000
1		×	×	×	×														<1
2		×	×	×	×														2000
3					×														1000
4						×													<1
5																			2000
1							×	×											2000
2										×	×								2000
3										×	×	×							2000
4											×	×							2000
5											×	×							4000
1																			2000
2																			500
3																			500
4																		×	×	2000
5																			4

Vaccine‡

Controls† Guinea Pig No.	Complement Fixation Titer at 21 Days
1	500
2	500
3	100
4	500
5	500

* Guinea pigs showing a temperature of 103.8 F or greater for 2 or more consecutive days were considered infected.

† Uninoculated guinea pigs were housed with the animals inoculated with living rickettsiae.

‡ Guinea pigs were inoculated with the vaccine diluent and housed in a separate area.

TABLE 3

Embryonated eggs inoculated with the Henzerling strain of Coxiella burnetii diluted in saline and in vaccine

Dilution of Infected Yolk Sac In:	1st Egg Passage		2nd Egg Passage		3rd Egg Passage	
	Deaths*	Smear	Deaths	Smear	Deaths	Smear
Saline						
10^{-7}	0/15	−†	15/15	+		
10^{-8}	0/13	−	15/15	+		
10^{-9}	0/13	−	15/15	+		
10^{-10}	2/15	−	12/14	+		
10^{-11}	3/13	−	5/15	+		
Vaccine						
10^{-7}	2/14	−	15/15	+		
10^{-8}	4/14	−	13/13	+		
10^{-9}	3/10	−	14/14	+		
10^{-10}	0/13	−	13/14	+		
10^{-11}	2/14	−	1/15	−	7/7	+
None	2/15	−	1/13	−	8/14	−
Uninoculated controls	0/15	−	3/14	−	0/15	−

* Fifteen embryonated eggs were initially inoculated and death between the 1st and 5th postinoculation days was attributed to trauma. The figures given in this column represented: (Number of embryos found dead between the 5th and 12th postinoculation days of incubation)/ (Total number of embryos alive on the 5th post-inoculation day).

† Yolk sacs from eggs found with dead embryos during the 12 day postinoculation incubation period as well as from surviving eggs were examined for rickettsiae.

increased sufficiently in yolk sacs (Smadel, 1956) to be visible under the microscope. In the guinea pigs, there were approximately 50 per cent fewer febrile animals with each of the dilutions in vaccine than with the corresponding saline dilutions. Evidently about 50 per cent of the guinea pigs were sufficiently immunized by the vaccine diluent to resist infection by the small number of live rickettsiae present, thereby reducing the reliability of temperature responses in guinea pigs for safety test purposes. Guinea pig complement-fixation titers as an indicator of the presence of live rickettsiae in vaccine also appeared to have the same limitations as reliance on temperatures in that responses at the 10^{-10} rickettsiae-vaccine dilution approached those of the group which received only vaccine.

It appears from the data obtained in studies with Q fever vaccine at this laboratory, as well as from the data of other workers (Ransom and Huebner, 1951; Malloch and Stoker, 1952; Sokolova and Fedorova, 1958) with *C. burnetii* that guinea pigs are not as sensitive nor as reliable as embryonated eggs in detecting minimal numbers of live rickettsiae. The embryonated egg has several other advantages over guinea pigs as a means of safety testing Q fever vaccine. Larger volumes of vaccine can be tested per individual egg than with guinea pigs without toxic reactions, and greater numbers of eggs can be handled more conveniently, permitting examination of larger volumes of vaccine.

From the results and experiences of these studies, a procedure for safety testing Q fever vaccine was developed. A representative sample of Q fever vaccine was centrifuged at 4 C for 2 hr at 22,620 × G. The supernatant was discarded and the sediment resuspended to $\frac{1}{10}$ the original volume with physiological saline containing 500 units of penicillin per ml. Five-tenths ml of this 10× concentrate was inoculated into the yolk sacs of 7-day-old embryonated eggs (1st egg passage), using one syringe for not more than 10 eggs (to reduce the possibility of passage of contamination inherent in eggs). The eggs were incubated for 12 days at 36 C and candled daily after the 5th day.

TABLE 4

Safety test of Q fever vaccine in embryonated eggs

Egg Passage	Control		Lot A		Lot B	
	Deaths*	Smear†	Deaths	Smear	Deaths	Smear
1st	0/15	−	2/9	−	2/14	−
2nd	1/8	−	1/9	−	1/8	−
3rd	0/12	−	1/9	−	11/11	+

* Fifteen embryonated eggs were initially inoculated and death between the 1st and 5th postinoculation days was attributed to trauma. The figure given in this column represented: (Number of embryos found dead between the 5th and 12th postinoculation days of incubation)/(Total number of embryos alive on the 5th postinoculation day).

† Yolk sacs from eggs found with dead embryos during the 12-day incubation period as well as from surviving eggs were examined for rickettsiae.

1960] TEST FOR Q FEVER VACCINE Impression smears of yolk sacs from eggs found with dead embryos during the incubation period were examined microscopically for rickettsiae and bacteria, and all such yolk sacs were discarded. From LD_{50} determinations of infected yolk sac membranes it was known that deaths of embryos in this time period could not be due to small numbers of rickettsiae and there could be little value in the passage of the causative factor of these nonspecific deaths. Furthermore, utilization of sufficient numbers of eggs in this 1st egg passage would still ensure surviving eggs with enough total inoculum to represent an adequate sampling of any particular lot of vaccine. On the 12th day after inoculation, all surviving eggs were harvested, the yolk sacs pooled, and samples of the pool examined for rickettsiae and bacteria. The pools were then made to 30 per cent suspensions with saline-penicillin solution by grinding for 2 min in a Waring Blendor safety cup, and samples were withdrawn for passage into embryonated eggs (2nd egg passage). The eggs were again incubated for 12 days and yolk sacs of both dead and surviving eggs examined microscopically for rickettsiae. The yolk sac pool of the surviving eggs was processed as described for the 2nd egg passage and 0.5 ml of the resultant suspension inoculated into eggs (3rd egg passage). If, after this passage, final confirmation was questionable, a 4th passage was made and the yolk sac pools of either the 3rd or 4th passage were converted to antigen for specific identification in the complement-fixation test. The results of tests of two lots of vaccine are shown in table 4.

Lot A, with few significant deaths and no visible rickettsiae in yolk sac smears from living or dead eggs in the 3 passages, was considered safe for human use. However, in the 3rd egg passage of lot B, all embryos died by the 8th postinoculation day and rickettsiae were found in the yolk sacs. A 4th passage was made, 100 per cent mortality was again recorded and rickettsiae were visible in the yolk sac membranes. The yolk sacs of these eggs were processed by the method of Plotz et al. (1948) for preparing purified suspensions of rickettsiae and this preparation gave a positive titer when used as an antigen in the complement-fixation test.

SUMMARY

Embryonated eggs were found to be more sensitive than guinea pigs to small numbers of *Coxiella burnetii* in vaccine and also provided a more reliable system whereby comparatively large volumes of Q fever vaccine could be examined for residual live rickettsiae.

A method of safety testing Q fever vaccine in embryonated eggs is described in which no visible rickettsiae in yolk sacs of the 3rd passage eggs was considered satisfactory proof of vaccine safety.

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