Ecology of Rocky Mountain Spotted Fever. I. *Rickettsia rickettsii* Recovered from a Cottontail Rabbit from Virginia.*† (26581)

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Certain species of terrestrial mammals are known to be essential hosts for perpetuation of the ixodid ticks which are vectors of spotted fever rickettsiae. Strong presumptive evidence indicates that these mammals are probably important hosts of *Rickettsia rickettsii*, but actual recovery of the organisms from a native species of wild mammal has been confirmed only once in North America(1). Between May and December 1960, 10 wild cottontail rabbits, *Sylvilagus floridanus*, were trapped in various regions in Virginia and sent alive to the Walter Reed Army Inst. of Research. The present paper reports the recovery and characterization of a strain of *R. rickettsii* from one of these animals.

Methods. Each animal was bled for serum, spleen and liver were removed aseptically, and one-half of each tissue was used to prepare a 20% suspension in Snyder I diluent(2) containing 100 units penicillin and 20 μg streptomycin per ml. The remaining portions of whole tissue were stored at −70°C. Aliquots of the suspension were used for inoculation of guinea pigs, embryonated hens' eggs and blood agar slants. The guinea pigs were observed daily for 14 days for fever and for scrotal reaction. When guinea pigs developed fever (104°F or higher) for 2 consecutive days, a suspension of their blood, spleen and liver was passed to 3 guinea pigs. If the guinea pigs showed no fever or scrotal reaction by the 10th day, one was sacrificed, and its tissues passed to 3 guinea pigs. All surviving guinea pigs were bled 21-28 days after inoculation in order to test their sera for presence of complement-fixing antibodies employing spotted fever group-reactive antigens and, in some instances, specific washed rickettsial antigens (3).

The eggs were candled daily for 12-14 days, and the yolk sac of each embryo found dead after the second day was harvested aseptically, smeared and stained by Macchiavello's method. If rickettsia-like bodies were noted, the yolk sac was passed to eggs. The yolk sac of any dead embryo which had no sign of infection, *i.e.*, no visible rickettsia-like organisms, was passed to more eggs because of the possibility that the number of organisms was so meager that they could not be found by routine microscopic examination. If none of the embryos died by the 10th-12th day, a "blind" passage of one of the yolk sacs was made to other eggs. An isolation attempt in eggs and guinea pigs was not considered negative until 3 serial passages had been performed.

Guinea pigs which had recovered from a suspected rickettsial infection were challenged with the Bates strain of *R. rickettsii*. (The Bates strain was recovered in 1956 in this laboratory from a human case of Rocky Mountain spotted fever.)

Results. Data on attempts to recover *R. rickettsii* from cottontail rabbits trapped in Virginia are summarized in Table I. A suspension prepared from the spleen and liver of Rabbit 1, inoculated into the yolk sacs of embryonated eggs, caused death of 3 of 6 embryos on the 8th day. Organisms resembling rickettsiae were seen in smears of the yolk sacs, and this material served to establish the strain in serial egg passage. One of the 2 guinea pigs originally inoculated with the rabbit material had fever on the 6th day. A sus-

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TABLE I. History and Laboratory Studies of Cottontail Rabbits *(Sylvilagus floridanus)* from Virginia (1960).

Rabbit No.	Locality trapped	Date trapped	State of maturity	Serum titer*	Recovery of *R. rickettsii*
1	Farmville	6 May	Juvenile	<5	+
2	Leesburg	23 June	"	320	0
3	Charlottesville	3 July	"	<5	0
4	"	10 July	"	<5	0
5	"	16 July	"	<5	0
6	Camp Pickett	18 Aug.	"	20	0
7	Covington	18 Aug.	Adult	40	0
8	Camp Pickett	25 Aug.	Juvenile	<5	0
9	" "	13 Oct.	Adult	10	0
10	Maurertown	9 Dec.	"	<5	0

* Reciprocal of dilution of serum reacting with 4 units of group-reactive spotted fever antigen to fix complement.

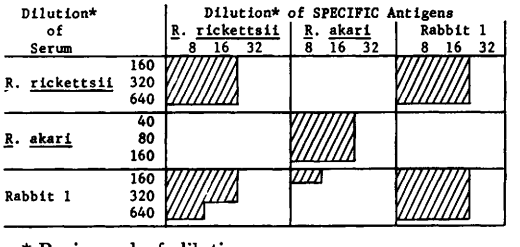
pension of blood, spleen and liver from this animal, sacrificed on the 2nd day of fever, served to establish the agent in serial guinea pig passages.

Behavior in guinea pigs: Following inoculation with established passage material, guinea pigs developed fever (104°F-105°F) which commenced on the 5th or 6th day, and persisted through the 10th or 11th day. None of the animals died, and only occasionally did any of them exhibit a grossly detectable tunica reaction manifested by definitely demonstrable adhesions.

Behavior in embryonated eggs: Standardized suspensions prepared from infected yolk sacs contained $10^{6.5}$ egg LD_{50}s when 0.2 ml of serial 10-fold dilutions were titrated in 7-day-old embryonated eggs. The lower dilutions caused death of the embryos in 4-5 days. Stained smears prepared from the yolk sacs usually contained 1-10 organisms/oil immersion field. Such infected yolk sac suspensions had infectious titers of about 10^{-7} when titrated in guinea pigs; animals which had 2-3 consecutive days of fever were considered to be infected since their convalescent sera invariably fixed complement in the presence of spotted fever group antigen.

Serologic behavior: Sera from guinea pigs which had been infected with the Rabbit 1 agent fixed complement in the presence of 2-4 units of group-reactive antigen prepared from yolk sacs infected with the Bitterroot strain of *R. rickettsii* or the 246 strain of *R. sibericus*. The Rabbit 1 guinea pig antiserum failed to react with 4 units of epidemic-murine typhus group antigen, scrub typhus, or Q fever antigen.

Group-reactive antigens prepared from yolk sacs infected with the Rabbit 1 agent fixed complement in the presence of convalescent sera from guinea pigs infected with the Rabbit strain, as well as *R. rickettsii* (2 strains), *R. akari, R. conorii* (3 strains), *R. australis, R. sibericus,* and the agents of maculatum disease and Indian tick typhus. Rabbit 1 antigens did not fix complement in the presence of sera from animals immune to *R. prowazeki, R. mooseri, R. tsutsugamushi* or *R. burneti.* Thus, the behavior of the new agent in complement fixation tests identified it as a member of the spotted fever group of rickettsiae. Moreover, specific washed rickettsial antigens prepared from yolk sac-propagated *R. rickettsii, R. akari,* and the Rabbit 1 agent distinguished the latter from *R. akari,* and identified it as a strain of *R. rickettsii* (Fig. 1). None of these specific antigens reacted with convalescent sera from guinea pigs infected with *R. sibericus, R. conorii, R. australis* and maculatum disease.



* Reciprocal of dilution.

FIG. 1. Results of reciprocal cross complement fixation tests employing specific washed antigens.

TABLE II. Results of Cross-immunity Tests in Guinea Pigs: Rabbit 1 *vs R. rickettsii, R. akari* and Maculatum Agent.

Immunizing strain*	Guinea pigs immune† when challenged with:			
	Rabbit 1	*R. rickettsii*	*R. akari*	Maculatum
Rabbit 1	6/6	6/6	6/6	6/6
R. rickettsii	6/6	6/6	—‡	—
R. akari	0/6	—	6/6	—
Maculatum agent	0/6	—	—	6/6
None (control)	0/4	0/4	0/4	0/4

* Standard strains and sources: *R. rickettsii*—Bates, a human case; *R. akari*—MK, from human case, New York; Maculatum agent—from tick, *Amblyomma maculatum*.

† Immune: No fever or scrotal reaction on challenge.

‡ Not done.

Cross-immunity: Table II summarizes a guinea pig cross-immunity test using the Rabbit strain and those agents of the spotted fever group known to occur naturally in North America. Initial infection with the Rabbit 1 agent protected all of the animals against a challenge with 100 guinea pig ID_{50}s of *R. rickettsii, R. akari,* maculatum disease and the homologous agent. Furthermore, those animals infected with *R. rickettsii* resisted challenge with the Rabbit 1 organism. On the other hand, an initial infection with *R. akari* or maculatum disease did not protect against a Rabbit 1 challenge. Thus, there was a one-way cross-immunity, *i.e.,* Rabbit 1 infection protected against challenge with maculatum disease or *R. akari,* but these agents did not immunize guinea pigs to infection with the Rabbit strain.

Behavior in mice: Intra-abdominal inoculation of albino mice with 10% Rabbit 1-infected yolk sac suspension failed to cause any apparent disease. The mice, however, were protected against challenge with 100 lethal infectious doses of the Hartford strain of *R. akari.*

Intravenous injection of infected yolk sac suspensions in concentrations of 5% or greater caused death of 10-12 g mice in 24 hours. Thus, the Rabbit strain has a toxic effect for mice similar to that reported by Bell and co-workers for their 3 strains of spotted fever rickettsiae(4).

Re-isolation: Although there was no doubt as to the validity of rabbit tissues as the source of the Rabbit 1 strain, the original stored tissues were thawed and tested, and a second line of the agent was recovered in eggs and guinea pigs inoculated with this material.

Discussion. The recovery of a strain of rickettsiae from a specimen of a species of wild mammal genus, long suspected to be a natural host for *R. rickettsii* in North America, not only warrants the fullest possible characterization of its biological properties, but also provides concrete evidence for the potential role of cottontail rabbits as hosts of *R. rickettsii* and possible donors of these organisms to ticks.

This strain of rickettsia is classified as *R. rickettsii* on the basis of the following observations. Its morphology is entirely consistent with that of rickettsiae. It grows intracellularly and fails to multiply within media containing no living cells. It produces a transmissible infection in guinea pigs, but fails to produce overt infection in adult albino mice, although the latter may succumb to the toxic effect of sufficiently large doses of viable organisms. Its behavior in cross-immunity tests in guinea pigs distinguishes it from known North American agents of the spotted fever group, other than *R. rickettsii.* Its serologic behavior in complement fixation provides the crucial evidence which (a) classifies it as a member of the spotted fever group and (b) identifies it as *R. rickettsii* when specific washed rickettsial antigens are prepared from it and tested against appropriate antisera. Likewise, infection with this agent produces in guinea pigs antibodies whose behavior in complement fixation identifies the agent as *R. rickettsii.* Although reciprocal cross-neutralization of toxicity for mice might provide further evidence of the identity of this organism, the observations cited above are considered to be sufficient.

With respect to the epidemiology of spotted

fever, it is of interest to note that the behavior of this strain in guinea pigs is not unlike that of some strains of *R. rickettsii* recovered from human cases of the disease. The implications are obvious. With reference to cottontail rabbits, Jellison(5) noted the highly significant degree of correlation between the geographic distribution of species of *Sylvilagus* and the occurrence of human cases of spotted fever in the United States. Furthermore, *Sylvilagus* spp. are frequently hosts for the immature stages of *Dermacentor variabilis* and *D. andersoni*, which, as adults, may transmit spotted fever to man.

In reporting their recovery of *R. rickettsii* from a naturally infected meadow mouse, *Microtus pennsylvanicus*, taken in Virginia, Gould and Miesse(1) discussed the possible significance of their finding and reviewed pertinent prior studies of previous workers in the United States and Latin America.

In the course of the present study, rickettsial strains have also been recovered from 1 of 330 *Peromyscus leucopus* and 1 of 2 *Pitymys pinetorum*. Although their behavior in complement fixation classifies these strains to be members of the spotted fever group, neither has yet been sufficiently characterized to permit precise species identification.

Serologic studies revealed the presence of complement-fixing antibodies for the spotted fever group in 4 of the 10 wild rabbits (Table I). In addition, a limited survey of the sera of certain other wild mammals revealed similarly reacting antibodies in the sera of 1 or more specimens of red fox, gray fox, opossum, raccoon, white-footed wood mouse, and deer. These findings are interpreted tentatively as evidence of past spotted fever infection.

Further studies are necessary in order to evaluate more precisely the role of other mammals as well as rabbits in the ecology of *R. rickettsii*. Current field and laboratory studies are thus aimed at answering such questions as the following: What is the most sensitive method for detecting present or past infection with *R. rickettsii* in a particular species of mammal? Can one recover viable *R. rickettsii* from mammals whose serum contains complement-fixing antibody? For how long a period of time can an individual mammal serve as a donor of *R. rickettsii* to non-infected ticks that feed upon it? Is there a relationship between age of a rabbit and time that it usually becomes infected?

Summary. From a naturally infected wild cottontail rabbit, *S. floridanus*, trapped in Virginia, an organism identified as *R. rickettsii* has been recovered and characterized. The role of cottontail rabbits and certain other native wild mammals in the ecology of Rocky Mountain spotted fever is discussed briefly in the light of this and other related findings.

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Serological Comparisons Between Infectious Canine Hepatitis Virus and Human Adenovirus Types. (26582)

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The adenovirus group is a large family of infectious agents which possesses certain common biological properties, including a soluble group-reactive complement-fixing antigen(1,2). Recent studies have disclosed biological similarities between infectious canine hepatitis virus (ICHV) and certain members of the human adenovirus group(3,