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## HUMORAL IMMUNE RESPONSE OF COTTONTAIL RABBITS NATURALLY INFECTED WITH *FRANCISELLA TULARENSIS* IN SOUTHERN ILLINOIS

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**ABSTRACT:** Cottontail rabbits (*Sylvilagus floridanus*) usually are thought to succumb to infection with *Francisella tularensis*. Reports of a rabbit population from southern Illinois (USA) with a high prevalence of *F. tularensis* antibodies suggested that some cottontails survived infection with this typically fatal bacterium. Our goal was to examine the humoral response of cottontails from a study area in southern Illinois for which multiple serum samples existed. Multiple sera were collected from 79 cottontails from 1986 to 1990 and 63% gained, lost, or maintained ELISA titers of IgM and IgG isotype antibodies. The typical pattern of antibody response appeared to be IgM isotype antibodies first, followed by IgG isotype antibodies, with both generally increasing to high titers. Negative culture attempts of liver tissue from 51 cottontails with varying antibody responses suggested that chronic infection did not occur in rabbits that developed antibody. The significance of the cottontail antibody response in resolution or prevention of tularemia infection remains unclear.

**Key words:** Cottontail rabbits, *Francisella tularensis*, humoral immune response, *Sylvilagus floridanus*, tularemia, zoonosis.

### INTRODUCTION

Tularemia may be an important mortality factor in cottontail rabbit (*Sylvilagus floridanus*) populations and has caused widespread epizootics (McCahan et al., 1962) in this host. The paucity of cottontails reported to be seropositive to *Francisella tularensis* has traditionally been perceived to be the result of exposure usually concluding as fatal disease (Jellison et al., 1961). Investigators have detected seropositive cottontails using tube agglutination techniques (Burgdorfer et al., 1974; Jacobson et al., 1978), and McKeever et al. (1958) reported one apparently healthy cottontail rabbit with an agglutination titer of 1:320. Woolf et al. (1993) used an enzyme-linked immunosorbent assay (ELISA) and reported cottontail anti-*F. tularensis* seroprevalences of 44% ( $n = 722$  tested) and 23% ( $n = 805$  tested) for IgM and IgG antibodies, respectively, in a semi-isolated population in southern Illinois from 1986 to 1990. These high seroprevalences suggested that many cottontails on

their study area had survived exposure to *F. tularensis*.

We examined serum from cottontails that had been captured and released multiple times on the same study area and during the same time period reported by Woolf et al. (1993). These multiple sera provided a unique opportunity to examine the humoral response of cottontails naturally infected with *F. tularensis*. The only other comparable data for cottontails was reported for six cottontails by Lepitzki et al. (1990) who found that cottontails gained, lost, and maintained titers over at least an 8 mo period.

### METHODS

Blood was collected from cottontail rabbits at Wayne Fitzgerald State Park (WFSP) (38°06'N, 88°56'W), Jefferson and Franklin counties (Illinois, USA) from 1986 to 1990 during live-trapping efforts or controlled hunts. Live-trapped rabbits were anesthetized with ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, New Jersey, USA) (40 mg per kg body weight) and 3 to 5 cc blood was taken via cardiac puncture. Blood was centrifuged for 5

min at 3,000 rpm or refrigerated overnight prior to removal of serum. An ELISA modified from Viljanen et al. (1983) was performed as described by Woolf et al. (1993) to examine cottontail sera for *F. tularensis* IgM and IgG antibodies. Sera with titers of  $\geq 1:80$  or  $\geq 1:100$  (depending on the dilution scheme) were considered positive, and serum dilutions were continued to 1:2,560.

Livers from 61 cottontails collected at WFSP from 1987–1990 were submitted to the Centers for Disease Control (Fort Collins, Colorado, USA) for culture of *F. tularensis*. Triturated liver specimens (2 to 5 g) were inoculated subcutaneously into white laboratory mice which were then monitored for 21 days. Mortality of mice which yielded cultures of *F. tularensis* occurred 3 to 5 days postinoculation, which is consistent with the hypothesis that these were fully virulent type A isolates of *F. tularensis*. Cultures were confirmed using a direct fluorescent antibody test. Briefly, a small amount of culture growth was smeared on a microscope slide, air-dried, and heat-fixed. Each smear was then covered with a small amount of *F. tularensis* fluorescein isothiocyanate (FITC)-labeled globulin (Centers for Disease Control, Atlanta, Georgia, USA) and incubated 30 min at room temperature. Slides were then washed briefly in phosphate buffered saline (PBS) followed by a 10 min soak in PBS, rinsed in distilled water, and air dried. Slides were examined with a fluorescent microscope and positive *F. tularensis* cultures appeared as brightly fluorescing yellow-green cocco-bacilli.

## RESULTS

Multiple serum samples (2 to 5) were obtained from 79 cottontails. Twenty-nine cottontails (37%) were initially negative for both IgM and IgG isotype antibodies and remained negative at the last sample [mean time between samples  $\pm$  SD = 87  $\pm$  96 days; range ( $r$ ) = 5 to 361 days].

Multiple samples from seven cottontails were negative on all samples for IgG, but seroconverted from IgM negative to positive. The shortest interval between seroconversion from IgM negative to positive was 21 days. Five rabbits converted to a titer of 1:160 (185  $\pm$  159 days;  $r$  = 21 to 393 days), one rabbit converted to 1:100 (26 days after initial sample) and then to 1:320 (114 days after initial sample), and one rabbit converted from IgM negative to

a titer  $\geq 1:2,560$  with a 38-day span between samples.

One cottontail that was initially negative for IgG antibodies but IgM positive at 1:640 showed an IgG titer of 1:160 269 days later and the IgM titer had increased to 1:1,280. This cottontail retained both IgM and IgG titers of 1:160 and 1:100, respectively, 88 days later.

Multiple serum samples were obtained from 19 rabbits in which all samples were positive for both IgM and IgG antibodies. Five of these 19 cottontails (26%) maintained relatively high ( $\geq 1:1,280$ ) IgG titers over a mean duration of 169  $\pm$  125 days ( $r$  = 32 to 277 days); IgM titers also remained high ( $\geq 1:640$ ) in three of the same five animals, and moderate in two animals (1:320 to 1:640). Low-to-moderate IgG and IgM titers were maintained by seven of the 19 rabbits for a mean duration of 51  $\pm$  38 days ( $r$  = 27 to 118 days). Three rabbits had falling ( $\geq 4$ -fold decrease) IgG titers over a mean duration of 169  $\pm$  169 days ( $r$  = 57 to 364 days), while IgM titers remained steady at low-to-moderate levels. The 387 day interval between the first and last samples of one cottontail (four samples total) illustrates that both IgM and IgG antibodies can persist for periods  $> 1$  yr. The endpoint titers for this animal initially were  $\geq 1:2,560$  for both antibody isotypes, but had decreased to 1:160 for both isotypes at day 387.

Two cottontails initially were negative for both IgM and IgG antibody, but were positive for both IgM and IgG 37 and 398 days later. Three cottontails that initially tested positive for both IgG and IgM at low titers ( $\leq 1:320$ ) subsequently lost the IgG titer (time between samples was 50, 116 and 308 days).

One rabbit initially tested positive for IgG ( $\geq 1:2,560$ ) and was IgM negative, but 52 days later had titers of 1:640 for IgG and 1:320 for IgM, and 229 days later had an IgG titer of 1:320 but was IgM negative. Five additional animals that initially were IgG negative but IgM positive at low titers (1:100 to 1:160) subsequently lost

the IgM titer (mean time between last IgM positive sample and first IgM negative sample =  $195 \pm 164$  days;  $r = 32$  to 293 days).

Five serum samples were obtained from one cottontail over a period of almost 3 yr, although IgM data was incomplete due to the small amount of sera available for testing. This animal initially had an IgG titer of 1:160 (IgM data unavailable), was IgG positive (1:160) 36 days later (IgM data unavailable), was IgG and IgM positive (both 1:320) 313 days following the first sample, and was still IgG positive (1:100) 426 days after the first sample (IgM data unavailable). The cottontail was negative for both IgG and IgM 1,076 days following the initial sample.

Another cottontail from which five sera were obtained over a 4.5-mo period tested IgG negative on all five sera, but converted from IgM negative to IgM positive (1:100) 26 days after the initial sample. This cottontail remained IgM positive 33 and 114 days after the initial sample at 1:100 and 1:320, respectively, but was IgM negative 137 days after the first sample was taken.

Livers from eight cottontail rabbits found dead or killed by hunters yielded pure cultures of *F. tularensis*; two additional specimens also produced mortality in mice and gave positive fluorescent antibody tests for *F. tularensis*, but the agent could not be separated from *Proteus* spp. contaminants. Two of the eight cultures came from cottontails that exhibited clinical signs of disease. One of these animals was radio-tagged and upon being approached was observed sitting upright with its head drooping downwards. This animal made no attempt to escape and was picked up by hand and placed in a wooden box trap for further observation. This animal died 2 hr postcapture. Another cottontail from which a positive culture was obtained had difficulty running according to the hunter who harvested it (Woolf et al., 1993). The remaining isolates came from cottontails which were radio-tagged and found dead during routine locations, dur-

ing routine trapping efforts, or by park personnel.

Serum was obtained shortly after death from four cottontails whose livers yielded cultures of *F. tularensis*. Three of the four were negative for both antibody isotypes at the time of death, although one of these three was positive for IgM (1:100) but negative for IgG antibody 124 days prior to death. One of the four tested positive for IgM at a titer of 1:160 4 days prior to death and had an IgM titer of 1:160 when found dead. Four additional cottontails from which cultures of *F. tularensis* were obtained had sera available from previous captures. One animal tested negative for both IgM and IgG 13 and 48 days prior to death, and another tested negative for both antibody isotypes 11 days prior to death. One cottontail was positive for IgM (1:100) 9 days prior to death but was IgG negative, and another was negative for both IgM and IgG 350 days prior to death but had an IgM titer of 1:160 41 days prior to death.

Endpoint titers for both IgM and IgG antibody were determined for 43 of 51 cottontails whose livers did not yield isolates of *F. tularensis*. Sera from 23 of the animals were negative for both IgM and IgG antibody. Sera from sixteen rabbits tested IgG negative but were IgM positive at titers of 1:100 to 1:2,560, suggesting that these animals were in the early stages of developing an antibody response. Four rabbits were positive for both IgM and IgG antibodies at titers of 1:100 to 1:640. The negative culture attempts for these 43 cottontails which were positive for *F. tularensis* antibodies suggests these animals had cleared *F. tularensis* from the liver shortly after exposure or infection.

#### DISCUSSION

Major limitations to understanding the cottontail humoral response in this study are not knowing the number of occasions or the most recent specific time that animals were exposed to *F. tularensis*. However, some clues can be gained as to the

general pattern of IgM and IgG antibody production. Overall IgM and IgG seasonal seroprevalence for cottontails at WFSP from 1986 through 1990, as reported by Woolf et al. (1993) was high, with 44% testing positive for IgM ( $n = 722$  tested) and 23% for IgG ( $n = 805$  tested) antibodies. If we assume that the humoral response of rabbits to *F. tularensis* progressed in a "normal" fashion with an initial rise in IgM antibody followed by a rise in IgG antibody, and lasted for considerable periods of time as reported for human tularemia (Carlsson et al., 1979), then there should have been few cottontails that tested positive for IgG and negative for IgM isotype antibodies. Data for both IgM and IgG antibody isotypes were available for 712 of the cottontails reported by Woolf et al. (1993). Only 4% of these 712 cottontails were IgG positive and IgM negative. However, 24% ( $n = 174$ ) were IgM positive but IgG negative, and almost 88% of these animals had relatively low IgM titers of  $\leq 1:320$ , which is consistent with the hypothesis that the IgM antibody titer was on the rise and that the IgG response had not yet begun. Almost 20% of the 712 animals tested positive for both antibody isotypes. Our data from multiple serum sampling showed seven animals that were negative on all samples for IgG but seroconverted from IgM negative to positive. An additional animal initially was seropositive for IgM but negative for IgG, seroconverted to IgG positive while maintaining an IgM titer, and maintained titers of IgM and IgG for at least 3 mo. These data taken together are consistent with the hypothesis that cottontails at WFSP exposed to *F. tularensis* generally responded initially with a rise in IgM antibodies, followed by a rise in IgG antibodies, with both isotypes increasing to relatively high titers.

Antibody titers for both IgM and IgG appeared to be long lasting in several cottontails, especially considering their relatively short life-span of only 2 to 3 yr (Lord, 1963). However, exposure and in-

fection of cottontails to *F. tularensis* does not always lead to high antibody titers. One cottontail from which a culture was obtained had a 1:160 IgM titer 6 wk prior to being found dead, at which time it was negative for IgM and IgG antibodies to *F. tularensis*.

The fact that significant numbers of cottontails in our study population had high and persisting *F. tularensis* antibody titers is somewhat surprising given the assumption of high mortality of infected cottontails. The role, if any, that *F. tularensis* antibodies play in prevention or resolution of tularemia infection in cottontails is not known. Cell-mediated immunity has been thought to provide the primary means by which protection against tularemia is conferred (reviewed by Tarnvik, 1989). However, a recent report has shown that serum antibody can protect against challenge in a mouse-*F. tularensis* live-vaccine strain model (Drabick et al., 1994). At this time we do not know what host or agent factors are responsible for the survival of WFSP cottontails exposed to and infected with *F. tularensis*.

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