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Source: Journal of Wildlife Diseases, 12(4) : 545-549

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-12.4.545

TYZZER'S DISEASE IN FREE-LIVING COTTONTAIL RABBITS (*Sylvilagus floridanus*) IN MARYLAND

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Abstract: Complement-fixing (CF) antibody to *Bacillus piliformis* antigen was found in 9 of 14 (64%) serum samples obtained from cottontail rabbits (*Sylvilagus floridanus*) killed in the wild. CF antibody was not present in the serum of 8 cottontail rabbits trapped as juveniles in the same geographic areas and held in captivity for 4 years. Sero-negative cottontail rabbits died acutely with lesions typical of Tyzzer's disease following the intragastric administration of $10^{3.8}$ ELD_{50} of *B. piliformis* spores. The possible influence of Tyzzer's disease upon the cyclic population pattern of cottontail rabbits in the wild is discussed. A hypothesis is presented that *B. piliformis* spores passed in the feces of diseased wild animals could contaminate pastures, hay and grain, and thereby serve as sources of infection to other animals.

INTRODUCTION

The first report of naturally-occurring Tyzzer's disease in a wild animal species was provided by Karstad *et al.* in 1971.[9] Significantly, they recognized a remarkable similarity between the lesions of Tyzzer's disease in muskrats (*Ondatra zibethica*) and those seen in Errington's disease. An apparently contagious fatal disease of uncertain etiology, Errington's disease is recognized as the cause of epizootics which decimate muskrat populations in Iowa and Wisconsin marshes and possibly snowshoe hares (*Lepus americanus*) in Saskatchewan.[10]

Tyzzer's disease was first described in 1917 as the cause of an acute fatal entero-hepatitis of Japanese waltzing mice. Epizootics of Tyzzer's disease in colonies of mice have since been observed in China, Sweden, England, Japan and the United States. Subsequent to the description of the spontaneous disease in laboratory rabbits in 1965,[1] Tyzzer's disease has been recognized in a variety of other animals maintained under laboratory conditions, including rats, gerbils, hamsters, cats[11,12] and rhesus monkeys. A review, including a description of the etiologic agent, *Bacillus piliformis,* has been published.[6] Most recently Tyzzer's disease was recognized as the cause of fatal disease in a dog and in a domesticated farm animal, the horse foal.[7,8,15,17] The diagnosis of Tyzzer's disease has been based upon histopathological procedures which demonstrate *B. piliformis* within the cytoplasm of affected cells. The agent has not been cultivated in cell-free media but has been isolated and successfully propagated in the yolk sac of embryonated hen's eggs.[2,5]

This report provides serologic evidence for the natural occurrence of Tyzzer's disease in wild cottontail rabbits (*Sylvilagus floridanus*) in Maryland and demonstrates the extremely susceptible nature of similar sero-negative captive cottontail rabbits to experimentally induced Tyzzer's disease.

MATERIALS AND METHODS

Serology

The micro-titer complement-fixation (CF) test (modified Kolmer technique)[13] was performed using 2 units of antigen and 2 exact units of complement. Preparation of the antigen, an aqueous liver extract of severely infected mice, has been described.[3] Vegetative phase of *B. piliformis* (rabbit origin), propagated in the yolk sac of embryonated hen's eggs,[5] was passaged 3 times in N.NIH(S) strain

weanling mice as a 10% mouse liver suspension in phosphate-buffered saline (PBS) pH 7.2 by intravenous inoculation (0.1 ml/mouse). The mice also received Cortone acetate (Cortisone Acetate, Merck Sharp and Dohme, Rahway, NJ), 2.5 mg subcutaneously, just prior to inoculation. Control antigen was prepared in a similar manner by passage of normal mouse liver suspension in cortisone stressed mice of the same shipments. Antigen was stored in flame-sealed ampoules at —70 C. The titer of antigen was 1:32, as revealed by box titration with a positive serum, and remained stable for at least 5 months. Control negative and positive sera were obtained by pre-bleeding 10-week-old New Zealand White rabbits, administering washed *B. piliformis* spores ($10^{3.8}$ ELD_{50} per rabbit) intragastrically via stomach tube, and bleeding 30-45 days later. Sera were collected[1] from wild cottontail rabbits (age unknown) killed or trapped in Frederick and Washington counties of Maryland during the fall of 1974 and stored at —20 C. Also, in the fall of 1974, sera were collected from 8 captive cottontail rabbits that were trapped in the wild as juveniles during the summer of 1971 in Allegany, Frederick and Montgomery counties of Maryland and maintained in a fenced, sheltered enclosure for 4 years at the National Institutes of Health Animal Center, Poolesville, MD. Pelleted ration and water were available *ad libitum*. The captive rabbits were subsequently moved to Bethesda, MD, caged individually in Horsfall-type units (under slight negative pressure), and used in transmission studies described below. Repeat serum samples were collected prior to inoculation.

Transmission studies

Six 4-year-old captive cottontail rabbits (described above) were used. Each of 4 rabbits received $10^{3.8}$ ELD_{50} of the 5th yolk sac passage of *B. piliformis* (rabbit origin) intragastrically (via stomach tube). Cortisone, 0.05 mg/g of body weight, was administered subcutaneously to 2 of the rabbits at the time of inoculation with *B. piliformis*. The remaining 2 uninoculated rabbits received comparable injections of cortisone. The inoculum was a 10% (w/v) saline (0.85% NaCl, pH 7.2) suspension of yolk sac harvested on the 5th post-inoculation day (PID) and stored at —20 C for 2 years. Quantitation of *B. piliformis* in embryonated eggs has been described previously.[5] Dead or moribund rabbits were necropsied and appropriate tissues were fixed in 10% unbuffered formalin. Stained tissue sections were prepared according to previously described technique[1] and examined histologically.

RESULTS

CF antibody was detected in sera obtained from 9 of 14 (64%) cottontail rabbits killed in the wild (Table 1). Comparable titers were obtained from sera of laboratory rabbits experimentally infected with *B. piliformis* (as early as 2 weeks and up to 4 months postinoculation, the longest time tested). CF antibody was not detected in any of 8 serum samples obtained from cottontail rabbits trapped as juveniles in the same geographic areas and held in captivity for 4 years.

Both of the sero-negative 4-year-old cottontail rabbits which received cortisone and *B. piliformis* passed an unformed tarry stool on the 3rd Post-inoculation day (PID) and were found dead in the cage on the morning of the 4th PID. The 2 rabbits which received *B. piliformis* alone passed a similar stool on the 3rd PID but in lesser quantity; one was found dead on the 5th PID, the other was moribund on the 6th PID and was killed. The perianal fur of each of these rabbits was soiled with tarry feces. Watery diarrhea and extensive soiling of the hocks, typical of Tyzzer's disease in laboratory rabbits,[1] were not seen. The 2 cottontail rabbits which received cortisone only remained healthy throughout a 2 week observation period.

[1] The authors wish to acknowledge the technical assistance of Mr. Russel P. Wiles, Jr., Small Animal Section, VRB, DRS, in obtaining the sera.

TABLE 1. Results of Complement Fixation Test.

	Negative	Serum titer 1:4	1:8	1:16	1:32
Cottontail rabbits killed as adults in the wild	5*	1	4	4	0
Cottontail rabbits captured as juveniles and held in captivity for 4 years	8	0	0	0	0
Laboratory rabbits given *B. piliformis* spores intragastrically					
Preinoculation	4	0	0	0	0
14 days	0	2	2	0	0
30 days	0	0	0	4	0
60 days	0	0	0	4	0
120 days	0	0	0	4	0

*Number of animals

At necropsy, the peritoneal fluid was moderately increased and straw-colored. Multiple scattered petechial hemorrhages were present in the serosa of the terminal ileum, cecum and proximal colon. Marked edema of the mesentery and visceral serosa at the ileo-cecal-colic junction, typical of the disease in laboratory rabbits,[1] was not observed. Careful examination of the liver revealed few miliary focal white spots scattered throughout the liver. A white band, 1 by 5 mm, was present in the myocardium of the left ventricle of the rabbit which survived to the 6th PID. Marked ulceration of the intestinal mucosa of the terminal 15 cm portion of the ileum, the cecum and proximal colon was conspicuously present in all 4 cottontail rabbits given *B. piliformis* spores.

The histopathology was similar to that seen in natural cases[1] and experimentally induced cases[5] of Tyzzer's disease in laboratory rabbits. The essential feature is focal necrosis of epithelial cells of the lower intestinal tract, hepatocytes, and occasionally of myocardial cells, and the demonstration of *B. piliformis* in the cytoplasm of apparently viable cells at the border of the necrotic cells.

DISCUSSION

The cottontail rabbit appears to be extremely susceptible to *B. piliformis* infection. The concurrent administration of cortisone, usually employed as an essential feature of experimentally induced Tyzzer's disease in laboratory mice and rabbits,[6] was not requisite to the induction of fatal Tyzzer's disease in the cottontail. The same dose of spores was non-lethal but infectious to young laboratory rabbits used for the production of positive serum used in the CF test. Furthermore, the cottontail rabbits used in this study were 4 years old. In the laboratory rabbit colony, fatal disease primarily occurs in young rabbits (5-8 weeks old); only rarely will the doe die even though her entire litter may die of Tyzzer's disease, a common event.[1]

Working with Tyzzer's disease in mice, Fujiwara[3] demonstrated the value of the CF test in studying the epizootiology of this infection in a colony. We found agreement in the preparation of antigen, the mechanics of the test, and in antibody levels obtained. However, we found CF antibody in 64% of the serum samples taken from cottontail rabbits killed

in the wild, while Fujiwara[3] found CF antibody in only 4 to 10% of the mice from infected colonies. To better understand the meaning of such a high rate, we need to know how long CF antibody persists in the cottontail rabbit which survives *B. piliformis* infection (unknown) and the age of the rabbits killed in the wild (unknown). In any event, such a high rate of exposure of wild cottontail rabbits to *B. piliformis* coupled with the extremely susceptible nature of the cottontail rabbit to experimentally induced infection suggests that Tyzzer's disease could be an important factor in explaining the cyclic pattern of the cottontail rabbit population in Maryland.[4]

Fuller[4] suggests that disease is probably the foremost cause of natural mortality in cottontal rabbits. Though the prevalence of disease is unknown, Fuller assumed that most die within dens or are consumed by predators while in a weakened condition. Interestingly, rarely does the cottontail live beyond the first year in Maryland even though the potential life span of this species exceeds 10 years. Furthermore, though losses to heavy hunting pressures are negligible, the population in some years is reduced by almost 90% only to fully recover the very next season. The present knowledge of Tyzzer's disease is compatible as an explanation for these observations. Thus it may be that Tyzzer's disease is responsible for major die-offs in wild cottontail rabbits as well as muskrats.

If, in the future, Tyzzer's disease in wild cottontal rabbits (and perhaps wild rodents) is found to be widespread in the United States and of common occurrence, it seems logical to expect unplowed pastures, hayfields, and runs and dens frequented by infected animals to be contaminated with the spores of *B. piliformis*. The survival and build-up of spores in such areas would provide an excellent source of infection to rabbits, rodents and foals, particularly if they eat dung. In the laboratory, spores of *B. piliformis* survive 23 C in dried yolk sac for at least 2 years and they are not adversely affected by repeated cycles of freeze and thaw.[5] The effect of sunlight on spore survival remains unknown.

Furthermore, hay cut from fields contaminated with feces from infected animals could be a source of infection to laboratory animals which are usually fed unpasteurized pelleted feed high in alfalfa meal content. Infected rodents which pass spore-laden feces also might contaminate stores of grain to be used in laboratory animal feed. Such a possibility could explain the spontaneous occurrence of Tyzzer's disease in a caesarean-derived SPF protected colony of mice.[14] Unfortunately, supporting data will be difficult to obtain because of the fastiduous intracellular requirement of isolation and cultivation of *B. piliformis* in the laboratory and the probable small number of spores in such large quantities of feed—somewhat like "looking for a needle in a hay stack."

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Received for publication 25 May 1976