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Source: Journal of Wildlife Diseases, 24(2) : 334-338

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-24.2.334

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Journal of Wildlife Diseases, 24(2), 1988, pp. 334–338
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LEPTOSPIRES IN THE MARINE TOAD (*BUFO MARINUS*) ON BARBADOS

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ABSTRACT: Leptospires were isolated from the kidneys of four of 211 toads (*Bufo marinus*) caught on Barbados. Two of the isolates were identified as *Leptospira interrogans* serovar *bim* in the Autumnalis serogroup (the most common cause of leptospiral illness on Barbados), and two as possibly new serovars in the Australis serogroup. Sera from 198 of the toads were examined by the leptospire microscopic agglutination test. Forty-two (21%) were positive at titers of ≥1:100, and 54 (27%) at ≥1:50. The predominating serogroups were Australis (50%), Autumnalis (23%) and Panama (13%). The agglutination tests on the culture-positive toads showed that serologic studies alone may be of limited value in these animals. *Bufo marinus* can harbor pathogenic leptospires, and it may be a significant source of the Autumnalis serogroup infections in the Caribbean.

Key words: Leptospires, marine toad, *Bufo marinus*, Caribbean, leptospire microscopic agglutination test, serological survey, *Leptospira interrogans* serovar *bim*.

INTRODUCTION

The Giant or Marine toad (*Bufo marinus*) is the most widespread and common amphibian in the new world, with a range extending from 27°N in southern Texas and western Mexico to 10°S in central Brazil. It lives in a variety of habitats, most commonly in and around human settlements, in open areas resulting from man's activity and in grasslands (Zug and Zug, 1979). There are no published ecological studies of *B. marinus* on Barbados since its introduction in approximately 1833 (Schomburgk, 1848), but our casual observations indicate that it is common in urban areas, gardens and agricultural land. Because of its close proximity to man, and because it lives in wet or damp places which favor survival of leptospires, its role in the epidemiology of leptospirosis is of interest. Leptospirosis is endemic on Barbados and causes severe human illness at the rate of 17.6 per 100,000 population per year (Everard et al., 1984). Little is known concerning the role amphibians have in spreading the disease (Minette, 1983), but *B. marinus* has been incriminated as a reservoir of *Leptospira interrogans* on Trinidad and Grenada (Everard et al., 1980, 1983) and in the Philippines (Babudieri et al., 1973). We report here on a serological and bacteriological study of *B. marinus* on Barbados undertaken intermittently between 1980 and 1986.

MATERIALS AND METHODS

Toads were captured in a variety of locations which were chosen haphazardly for the trappers' convenience. They were kept overnight in metal cans with damp vegetation, and were taken to the laboratory the next day where they were anesthetized in a jar with cotton wool soaked in diethyl ether. When moribund they were submerged in Cetrimide (Sigma Chemical Co., Poole, Dorset BH17 7NH, England) at a dilution of 1 in 10,000 for 5 min.

Blood and kidney tissue were cultured for leptospires according to the methods of Sulzer and Jones (1978). Up to 2 ml of blood was collected from the heart with a sterile needle, and 1 drop was placed into each of two bottles containing approximately 5 ml EMJH semisolid medium (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967). The remainder of the blood was set aside for serological examination. Both kidneys were then removed aseptically and sectioned together. A small piece was placed into each of two bottles of semisolid EMJH to which 0.05 ml of 1% 5-fluorouracil had been added; the remaining kidney material was put into liquid EMJH and left overnight to pass through a 0.22 μm Falcon filter (Becton Dickinson and Co., Cockeysville, Maryland 21030, USA). A few drops of the filtrate were also cultured in two bottles of EMJH semisolid medium. Cultures made after June 1984 were further enriched with 1% rabbit serum (Serological Reagents Ltd., East Grinstead, Sussex RH19 3YL, England). All cultures were kept in an air conditioned room with an ambient temperature of

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TABLE 1. The highest microscopic agglutination test titers and predominating *Leptospira interrogans* serogroups in 47 *Bufo marinus* sera which showed one predominating seroreactor.

Serogroup	Titer					Total	%
	1:50	1:100	1:200	1:400	1:800		
Australis	5	5	8	3	2	23	50
Autumnalis	—	3	6	2	—	11	23
Panama	1	2	2	—	1	6	13
Canicola	2	2	—	—	—	4	9
Icterohaemorrhagiae	1	1	—	—	—	2	4
Sejroe	1	—	—	—	—	1	2
Total	10	13	16	5	3	47	101

approximately 25 C. They were examined after 4 wk, and at 2-wk intervals thereafter for 4 mo. Isolates were identified at the Royal Tropical Institute (Amsterdam, Meibergdreef 39, The Netherlands) by cross agglutination absorption tests (Dikken and Kmety, 1978) and monoclonal antibody technology (Terpstra et al., 1985).

The remainder of each blood sample was centrifuged and the serum was examined by the microscopic agglutination test (MAT) according to standard methodology (Cole et al., 1973; Sulzer and Jones, 1978). The sera were titrated against the following 22 live culture serovar antigens: *ballum* and *arboreae* (local strain Lloyd) (serogroup Ballum); *canicola* (Canicola); *pyrogenes* (Pyrogenes), *copenhageni* (Icterohaemorrhagiae), *bataviae* and *brasiliensis* (Bataviae); *grippotyphosa* (Grippotyphosa); *fortbragg* and *bim* (Autumnalis); *pomona* (Pomona); *sejroe* and *hardjo* (Sejroe); *georgia* (Mini); *tarassovi* (Tarassovi); *panama* and *mangus* (Panama); *bratislava* and *australis* (Australis); *javanica* (Javanica); *cynopteri* (Cynopteri); and *patoc I* (from the saprophytic serogroup Semaranga). The sera were screened at 1 in 50 and 1 in 100, and positive sera were titrated to the end point.

RESULTS

A total of 211 toads was examined; these consisted of 20, 11, 9, 86 and 85 taken in 1980, 1982, 1984, 1985 and 1986, respectively. They were caught at 20 locations in various parts of the island of Barbados. Seventy percent of the toads came from the more densely populated hinterland of the south and west coasts, none were from the north, and only 13 were captured in the central hilly area which has the highest rainfall (>160 cm per yr).

Sera from 198 toads were tested by the MAT, of which 42 (21%) were positive at titers of ≥1:100. In an additional 12 toads the highest titer was 1:50 (27% positive at ≥1:50), while the remaining 144 had agglutinins at <1:50 or were negative. In 1985 and 1986 the proportions of toad sera positive at ≥1:50 were similar at 28% (23 of 83) and 30% (25 of 84), respectively. The numbers of sera examined in the other years (20, two and nine for 1980, 1982 and 1984, respectively) were too small to give meaningful percentages.

Forty-seven of the 54 sera which were positive at ≥1:50 reacted to only one serogroup antigen, or to one serogroup at a higher titer than to other serogroups. Among these 47 sera, the most commonly recorded predominating serogroups were Australis (50%), Autumnalis (23%) and Panama (13%) (Table 1). Table 1 also shows that the majority of sera reacted at 1:100 or 1:200, and that the highest titer was 1:800 (in three toads). Of the seven sera which reacted to ≥two serogroup antigens at the same titer, the predominating groups were Australis and Autumnalis (3), Australis and Panama (2), Autumnalis and Canicola (1) and Panama and Javanica (1).

Cultures were attempted from all 211 toads. Isolates were obtained from the kidneys of four of these. These animals were part of a group of 17 captured in the same area of suburban Bridgetown between 22 January and 13 March 1985. Those which yielded isolates came from the collector's garden. Two of the isolates were identified as Autumnalis *bim*, and two as possibly

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new serovars in the Australis serogroup (to be published elsewhere). The serology of the toads from which isolates were obtained was not a good indicator of infection or of the identity of the infecting serogroup. In only one toad (#60) was the infecting group reflected by the serology. Here, a serovar in the Australis serogroup was isolated, while the serum reacted to Australis *australis* at 1:200 and Panama *panama* at 1:100. The isolate from the second toad (#67) was also in the Australis serogroup, but the serum reacted to Australis *bratislava* at only <1:50 (this is normally classified as negative). In the third toad (#69) Autumnalis *bim* was isolated, while the serology was Australis *bratislava* 1:800, Australis *australis* 1:100, Autumnalis *bim* 1:400, Autumnalis *fort-bragg* 1:200, and Semaranga *patoc* <1:50. In the fourth toad (#99), which also yielded an isolate of Autumnalis *bim*, the serum was negative. Among the remaining 11 toads taken from the same area which were examined by the MAT, one had a titer of 1:200 to Australis, one had a titer of 1:50 to Sejroe, and nine were negative.

DISCUSSION

In a study undertaken between 1975 and 1979, Everard et al. (1983) found that 20 of 80 (25%) *B. marinus* on Trinidad and 10 of 66 (15%) on Grenada were seropositive to leptospiral antigens at titers ≥1:100. These figures are comparable to the 21% recorded in toads from Barbados. The major seroreactive serogroups in the toads from Trinidad were Autumnalis and Hebdomadis, followed by Tarassovi; in Grenada they were Hebdomadis, Icterohaemorrhagiae and Panama. Isolates were obtained from eight *B. marinus* from Trinidad and two from Grenada. The isolates from Trinidad were all identified as Autumnalis *autumnalis*, and the isolates from Grenada as Tarassovi *navet* and Australis *peruviana*. This was the first isolation of Australis in the southeastern Caribbean. Everard et al. (1980, 1983) point out that the predominating serogroup in toads from Trinidad (Autumnalis) was that to which agricultural workers were most commonly exposed, and that the density of these toads makes them an important reservoir of leptospires, especially those in the Autumnalis serogroup. The present study also indicates that *B. marinus* could be a significant source of Autumnalis infection in the Caribbean. Further, this toad's ability to harbor pathogenic leptospires is of importance also in Hawaii, Fiji, the Philippines, Australia and other countries to which it has been introduced.

The serogroup Australis is not known to cause severe leptospirosis on Barbados, either in man or in animals. The presumptive infecting leptospiral serogroups recorded between late 1979 and the end of 1986 in hospital patients from Barbados with leptospirosis were Autumnalis (*bim*) (61%), Icterohaemorrhagiae (19%), Ballum (11%) and Canicola (8%) (Everard, unpubl. data). In addition, Grippotyphosa, Panama and Australis were each incriminated once based on serological evidence alone. Altogether four patients had a serology in which Australis predominated; three of these yielded isolates of Autumnalis *bim* (Everard, unpubl. data). However, while the infecting serogroup of the fourth patient also was likely to be Autumnalis, cross-reactivity or paradoxical reactions can no longer be presumed following the recent isolation of possibly new serovars in the Australis serogroup.

Presently there is little evidence from other areas of the importance of toads in the epidemiology of leptospirosis, although this is in part due to the lack of attention that these animals have received as potential reservoirs for the infection. Minette (1983) reviewed leptospires in poikilothermic vertebrates and quoted studies by Cordeiro et al. (1981), Emmanuel et al. (1964) and Schnurrenberger et al. (1970) in which 22, 21 and one toad, respectively, were found to be negative by serology and/or culture. Babudieri et al. (1973) made three isolates from 112 *B. marinus* in the Philippines, none of which showed signif-

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icant agglutination titers. One isolate was lost and the other two, which were identical, were named *Bufonis carlos* (new serogroup and serovar). The serovar *carlos* has now been placed in the Autumnalis serogroup.

Our findings in this and the earlier study on Grenada and Trinidad (Everard et al., 1983) confirm the observation of Minette (1983) that serological studies as the sole source of evidence for leptospiral infection in poikilothermic vertebrates may be of limited value. Both of the toads from Grenada in our previous study which yielded isolates were serologically negative, as were two of eight in Trinidad. Four of the toads from Trinidad had serologies which corresponded to the infecting serogroup, but two were positive for a different serogroup (Icterohaemorrhagiae, which cross-reacts with Autumnalis) (Everard, unpubl. data). The existence of culture-positive seronegative toads is of interest. This commonly occurs in rodents, and it is generally considered that their ability to harbor leptospires in the kidney without eliciting an antibody response is indicative of long-term shedding of leptospires. Whether or not this applies to toads is not known. Evans et al. (1965) suggested that amphibians may lack the capacity for immunological memory and that antibody formation could be considerably delayed. They noted that the optimum temperature for maximum antibody synthesis in *B. marinus* was 25 C, and that temperatures 10 degrees above and below this optimum resulted in decreased antibody formation. However, since *B. marinus* hides under rocks and in crevices during the daytime, in Barbados its body temperature is unlikely to range beyond 25 ± 3 C. Therefore, temperature is unlikely to affect antibody formation.

Our culture success rate in *B. marinus* from Barbados (4/211 or 2%) was comparable to that in Grenada (2/58 or 3%) (Everard et al., 1983), but lower than that obtained in Trinidad (8/76 or 11%) (Everard, unpubl. data). The methodology used in the present study was essentially the same as that used to isolate leptospires from hospital cases in Barbados, where the success rate was 47% in 1985 and 1986 (Everard, unpubl. data). It may be that few toads harbor leptospires, or that special techniques are required to isolate them. The body temperature of *B. marinus* in Barbados is approximately that of the air conditioned room in which our cultures were kept (25 ± 2 C), so that temperature is unlikely to have inhibited leptospiral growth.

The fact that no ecological studies, particularly population estimates, of *B. marinus* have been undertaken in Barbados makes it difficult to assess the importance of this species as a reservoir for leptospires. It is a highly adaptable omnivorous species. It can live up to 16 yr, and at least in its adult stages has few predators because of its noxious parotid gland exudate and large size (Zug and Zug, 1979). There are few possible predators on Barbados other than rats and some bird species which probably mostly prey on young toadlets. Mongooses (*Herpestes auropunctatus*) will eat adult toads, but dogs and cats avoid the adults (Nellis and Everard, 1983). Dehydration and a shortage of insects in the dry season are significant mortality factors in *B. marinus* populations (Zug and Zug, 1979), and would be especially so in Barbados where there is little standing water in the dry season, and where cane fires and the widespread use of pesticides deplete insect populations. We feel that it is unlikely that *B. marinus* populations in Barbados will expand to the point where they are considered a pest, as may be the case in Queensland, Australia (Hughes, 1982).

Bufo marinus can absorb water through their skin (Zug and Zug, 1979). Water is stored in the urinary bladder and can be resorbed during dehydration stress. It is expelled when the toad is captured and subsequently handled, and for this reason we did not culture it. The capacity of the urinary bladder is probably near 30% of gross body weight (Zug and Zug, 1979), so

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that a large toad may expel a significant quantity of possibly infected liquid. The viability of leptospires shed by these toads may depend on seasonal variations in the amount and composition of the urine.

ACKNOWLEDGMENTS

This study was funded by the British Medical Research Council (MRC) and Overseas Development Administration, the Government of Barbados, and the Royal Tropical Institute, Amsterdam, The Netherlands. We are grateful to the Chief Medical Officer of Barbados for allowing it to be undertaken. We thank G. B. Webb, F. B. M. Rodrigues, C. Cummins, D. Jean-Marie, Don-Eyre Beckles, S. Daniel and E. Goodman of the MRC/Barbados Government *Leptospira* Laboratory, for additional technical assistance; and we acknowledge our special gratitude to Don-Eyre Beckles for providing the toads which yielded isolates.

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Received for publication 5 August 1987.

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