

# ISOLATION OF SEROTYPE *HARDJO* AND OTHER LEPTOSPIRAE FROM ARMADILLOS IN ARGENTINA<sup>1</sup>

Donald M. Myers,<sup>2</sup> Alberto Cuba Caparo,<sup>2</sup> and Jaime Payan Moreno<sup>3</sup>

*Thirteen pathogenic Leptospira interrogans strains and two saprophytic Leptospira biflexa strains were isolated from 89 armadillos (Chaetophractus villosus) in Argentina. This is the first time that isolation and identification of serotype hardjo from this animal species has been reported. Serologic, cultural, and histopathologic studies performed in the course of this work indicate that the armadillo is an important reservoir-host for leptospirae in Argentina.*

## Introduction

Wildlife populations widely infected with leptospirae are an important source of leptospirosis in domestic animals and man. The epidemiologic importance of such carrier-hosts results from their ability to contaminate the environment with urine containing large numbers of infective organisms. In this regard, continually changing ecological conditions have made it necessary to search out new host-reservoirs for ever increasing numbers of known pathogenic *Leptospira interrogans* serotypes.

In Argentina, the armadillo *Chaetophractus villosus* is abundant. The grass-land portions of the country that constitute its habitat are used extensively for raising cattle. The possible role of this armadillo as a potential natural reservoir for leptospirae was first demonstrated by Cacchione et al.

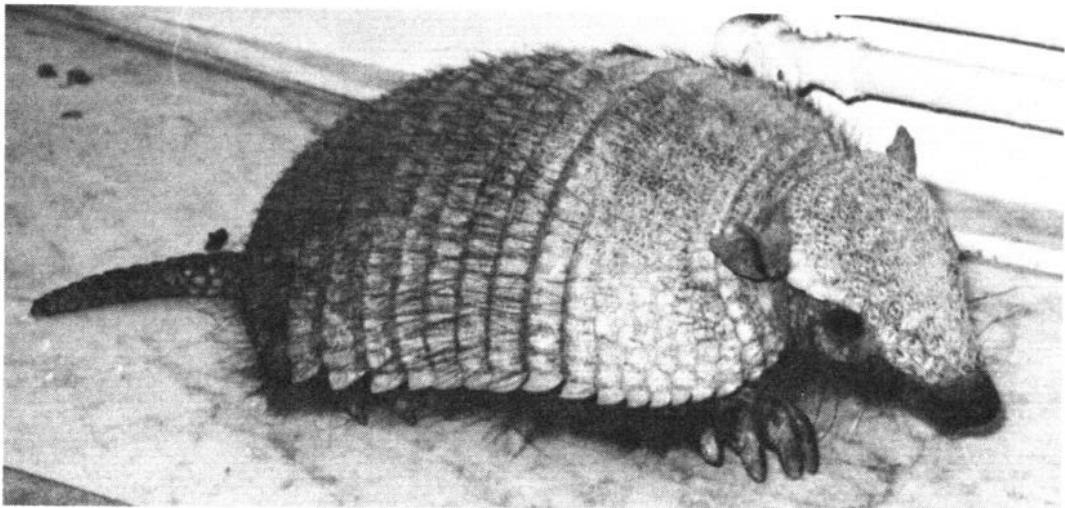
(1) through isolation of a *canicola* strain. Subsequently, Szyfres et al. (2) reported identifying and isolating two serogroup Bataviae serotypes (*paidjan* and *argentiniensis*) from armadillos; one of these serotypes (*paidjan*) was also obtained from a cow.

A more recent armadillo study by Carillo et al. (3) obtained 24 leptospiral isolates from 438 captured animals. All of these leptospirae proved to belong to Bataviae group serotypes *argentiniensis*, *paidjan*, or *bataviae*. However, examination of sera from the armadillos showed that their predominant antibodies were specific for the antigenically distinct *Hebdomadis* serogroup. Related serologic surveys of Argentine cattle have also found predominant agglutinins to the *Hebdomadis* group (4); these latter surveys made no cultural isolations. A later study, carried out on Argentine cattle by Myers and Jelambi (5) using improved culture techniques, succeeded in isolating six serotype *hardjo* strains of the *Hebdomadis* group from bovine kidneys. Taken together, these findings suggested that the armadillo could be either a reservoir or a maintaining host for bovine leptospirosis.

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<sup>2</sup>Leptospirosis and Pathology Units, Pan American Zoonoses Center, Pan American Health Organization, Casilla 3092, Correo Central, Buenos Aires, Argentina.

<sup>3</sup>Colombian Agriculture and Livestock Institute, Cali, Colombia.



The armadillo *Chaetophractus villosus*.

The purpose of the present study was to examine armadillos captured from selected cattle farms with culture techniques adequate for isolating all leptospira serotypes—including serotype *hardjo*. Histopathologic studies were also carried out on those armadillos from which isolates were obtained, in an effort to further determine whether the armadillo is an important chronic natural reservoir for leptospire or is merely an accidental host.

### Materials and Methods

The 89 armadillos examined in this study were hand-captured on four cattle-raising farms in the central area of the Province of Buenos Aires (Partido de Azul). They were captured in 13 batches of 6 to 10 animals each from March to July 1975.

Blood for serologic testing was collected from each animal by cardiac puncture. The animals were then killed by ether anesthesia, necropsied, and examined for gross pathology. An entire kidney of each animal was removed aseptically; one portion was used for cultural isolation of leptospire, and the rest was fixed in 10 per cent neutral formalin and embedded in paraffin. Tissue sections were cut and stained using both conventional staining methods (for histopathologic study) and Warthin-Starry silver stain (6) for detection of leptospire.

### Serologic Testing

The sera were subjected to the microscopic-agglutination (MA) test procedure described by Galton et al. (7). The antigens used in this test were live *Leptospira interrogans* serotypes grown in Stuart (Difco) liquid medium containing 10 per cent pooled normal rabbit serum. The specific strains used were as follows: *pomona* strain Pomona, *australis* strain Ballico, *ballum* strain Castellon 3, *Grippityphosa* strain Moskva V, *tarassovi* strain Perepelicin, *hebdomadis* strain Hebdomadis, *bataviae* strain Van Tienen, *canicola* strain Hond Utrecht IV, *hardjo* strain Hardjoprajitno, *wolffi* strain 3705, *paidjan* strain Paidjan, *argentiniensis* strain LT 1019, *pyrogenes* strain Salinem, *icterohaemorrhagiae* strain RGA, *sejroe* strain M 84, and *autumnalis* strain Akiyami A.

The sera were diluted with Sorensen phosphate-buffered saline (pH 7.2) to give final serum dilutions of 1:50 and 1:100. Those sera showing 50 per cent or more agglutination with the 1:100 dilution were further tested to determine their end-point titers, using two-fold serial dilutions. Equal volumes (0.2 ml) of standardized antigen suspension and diluted serum were used in all the MA tests, and readings were made after incubation for two hours at room temperature.

Serum titers equalling or exceeding 1:50 were considered seropositive in this study. Although serum titers under 1:100 are not routinely considered seropositive, negative MA titers were found for some animals from which leptospire were isolated—a finding which justified considering sera with lower titers positive.

### Culture Procedures

For culture isolation attempts, kidney tissue was triturated and suspended in nine parts of sterile phosphate-buffered saline (pH 7.2). Serial ten-fold dilutions of the tissue suspensions were then made with the sterile buffered saline, and 0.5 ml of each  $10^{-2}$  to  $10^{-4}$  dilution was used to inoculate duplicate tubes to which 5.0 ml of Fletcher semisolid medium (containing 10 per cent pooled rabbit serum) had been added. The same dilutions of renal tissue were also used to inoculate duplicate tubes containing 5.0 ml of bovine albumin-polysorbate 80 (BA-P80) medium—a medium using bovine fraction V albumin (Pentex) that was prepared as described by Johnson and co-workers (8, 9).

For contamination control, other sets of cultures were made by exposing a  $10^{-2}$  saline dilution of renal tissue to the combined action of both neomycin and furazolidone (at a final concentration of 25  $\mu\text{g}/\text{ml}$  each) for one hour prior to culture (10), after which  $10^{-3}$  and  $10^{-4}$  dilutions were made in antimicrobial-free saline. A portion (0.5 ml) of each dilution was then added to two tubes of Fletcher and two tubes of BA-P80 semisolid media.

Cultures were incubated at 30°C and examined weekly for eight weeks by darkfield microscopy to detect leptospire. When growth was detected, successive transfers were made in semisolid media until growth was sufficiently abundant for adaptation to liquid media. Further subcultures were made in Stuart or BA-P80 liquid media until sufficient growth was

obtained to make them satisfactory for use as antigen.

These leptospiral isolates were then tested against a battery of prepared hyperimmune rabbit antisera to pathogenic and saprophytic *Leptospira* serotypes in order to determine the serogroups of the isolates. Specific antisera were prepared for each isolate in accord with the methods of Galton *et al.* (7), and definitive identification was made of representative strains using the cross-agglutination-absorption procedures of Kmety *et al.* (11).

### Results

Serologic examination of sera from 89 armadillos showed that 42 (47.1 per cent) had antibody titers of at least 1:50. Thirty-one of these positively reacting sera (34.8 per cent of all sera collected) yielded MA titers ranging between 1:100 and 1:12,800 to one or more of the 16 leptospiral screening antigens. The 42 positive reactions and the predominant leptospiral agglutinins involved are shown in Table 1. As indicated, the most frequent reactions occurred against serotypes of the Hebdomadis and Bataviae groups, which together accounted for 78.6 per cent of the serologically positive animals.

Leptospire were isolated from renal tissues of 15 armadillos (16.8 per cent of those examined). Reciprocal cross-agglutination patterns against antisera to the different leptospiral serogroups showed that three of the isolates belonged to the Canicola group. In cross-agglutination-absorption tests these strains proved to be serologically homologous to serotype *canicola*. Nine of the other isolates were identified as members of the Bataviae group, and their similar cross-reaction patterns with type strains showed them to be antigenically identical to serotype *paidjan*, serotype *argentiniensis*, or serotype *bataviae*.

Another isolate (from armadillo No. 72) proved to be a member of the Hebdomadis

**Table 1. Prevalence of predominant leptospiral serum agglutinins in 89 armadillos captured on four cattle farms in Argentina.**

Antigen	Distribution of titers <sup>a</sup>					Total positive sera	
	1:50 to 1:100	1:200 to 1:400	1:800 to 1:1,600	1:3,200 to 1:6,400	1:12,800 or over	No.	%
Hebdomadis group <sup>b</sup>	14	4	1			19	21.3
Bataviae group <sup>c</sup>	2	3	4	4	1	14	15.7
Serotype <i>canicola</i>	1		1			2	2.2
Serotype <i>pomona</i>	1					1	1.1
Miscellaneous <sup>d</sup>	6					6	6.7
Total	24	7	6	4	1	42	47.1

<sup>a</sup>Reciprocal of the highest dilution showing 50 per cent or more agglutination.

<sup>b</sup>Hebdomadis group antigens: *hardjo*, *wolffi*, *sejroe*, and *hebdomadis*.

<sup>c</sup>Bataviae group antigens: *argentiniensis*, *paidjan*, and *bataviae*.

<sup>d</sup>Multiple reactions to two or more antigens at the same titer.

serogroup. Reciprocal-agglutination-absorption tests were performed with this isolate against various Hebdomadis group serotypes showing strong agglutinin activity. The results of these tests are presented in Table 2. On the basis of current taxonomic criteria, which limit residual titers to 10 per cent or less of the pre-absorption homo-

logous titers, the tests show that this isolate is serologically homologous to serotype *hardjo*.

The remaining two isolates did not react with any of the leptospiral antisera and showed characteristics compatible with saprophytic strains of the species *Leptospira biflexa*.

**Table 2. Results of cross-agglutination-absorption tests with the *Leptospira* isolate from armadillo No. 72 and selected serotypes of the Hebdomadis group.**

Antisera		Agglutination titer <sup>a</sup> with the indicated antigen			
Serotypes	Absorbed with	Homologous antigen		Antigen of absorbing culture	
		Before absorption	After absorption	Before absorption	After absorption
<i>hardjo</i> (Hardjoprajitno)	72	51,200	1,600	12,800	100
<i>wolffi</i> (3705)	72	51,200	25,600	25,600	200
<i>sejroe</i> (M 84)	72	25,600	25,600	6,400	—
<i>balcanica</i> (1627 Burgas)	72	12,800	3,200	12,800	200
<i>polonica</i> (493 Poland)	72	12,800	6,400	12,800	100
<i>trinidad</i> (LT 1098)	72	25,600	6,400	25,600	200
<i>gorgas</i> (LT 829)	72	12,800	6,400	12,800	100
72	<i>hardjo</i> (Hardjoprajitno)	12,800	400	12,800	—
72	<i>wolffi</i> (3705)	12,800	3,200	6,400	200
72	<i>sejroe</i> (M84)	12,800	6,400	1,600	400
72	<i>balcanica</i> (1627 Burgas)	12,800	1,600	3,200	—
72	<i>polonica</i> (493 Poland)	12,800	3,200	6,400	100
72	<i>trinidad</i> (LT 1098)	12,800	800	3,200	—
72	<i>gorgas</i> (LT 829)	12,800	6,400	3,200	—

<sup>a</sup>Reciprocal of the highest dilution showing 50 per cent or more agglutination.

— Indicates less than 50 per cent agglutination at a 1:100 serum dilution.

Table 3. Microscopic-agglutination titers obtained with positive sera (from 13 armadillos yielding pathogenic leptospire) when tested against various *Leptospira interrogans* serotypes.

Antigen	Titers <sup>a</sup> obtained with sera from each animal, by animal number <sup>b</sup>												
	1	2	4	58	60	62	64	65	72	73	74	77	81
<i>canicola</i>	—	—	800	—	—	—	—	—	—	—	—	—	100
<i>ballum</i>	—	—	50	—	—	100	—	—	—	—	—	—	—
<i>pyrogenes</i>	—	—	—	—	—	—	—	—	—	—	—	—	50
<i>bataviae</i>	1,600	—	—	—	—	400	—	3,200	—	—	3,200	100	—
<i>paidjan</i>	1,600	—	—	—	—	—	—	—	50	—	100	1,600	—
<i>argentiniensis</i>	3,200	—	—	6,400	400	6,400	200	12,800	1,600	1,600	6,400	800	—
<i>hardjo</i>	50	200	50	50	—	100	—	—	100	—	—	—	400
<i>wolffi</i>	—	—	—	—	—	—	—	—	—	—	—	—	400
<i>sejroe</i>	—	—	—	—	—	100	—	—	50	—	—	—	400
<i>hebdomadis</i>	—	—	—	—	—	—	—	—	50	—	—	—	100
<i>tarassovi</i>	—	—	—	—	—	50	—	200	—	—	50	—	—

<sup>a</sup>Titers expressed as reciprocal serum dilution against antigen.

<sup>b</sup>Animals with *Canicola* group strains: 2, 4, 81; with *Bataviae* group strains: 1, 58, 60, 62, 64, 65, 73, 74, 77; and with the *Hebdomadis* group strain *hardjo*: 72. No data are shown for two serologically negative animals yielding strains of *L. biflexa*. No reactions occurred with the following *L. interrogans* antigens: *autumnalis*, *grippotyphosa*, *pomona*, *australis*, and *icterohaemorrhagiae*.

Of the 13 animals from which pathogenic *L. interrogans* serotypes were isolated, 12 showed corresponding homologous group serum agglutinins with MA titers as high as 1:12,800 (see Table 3).

#### Autopsy Findings

Macroscopic examination of the kidneys, heart, spleen and skeletal muscles of all the autopsied animals revealed no significant pathological alterations. Two armadillos from which leptospire were isolated had enlarged livers showing marked fatty metamorphosis. Also, many of the animals had cyst-like formations 1 to 3 mm in diameter in their lungs. These latter incidental findings were diagnosed by microscopic examination to be unidentified parasitic nematode granulomas.

Histopathologic examination of the kidneys from 11 of the 13 armadillos yielding pathogenic leptospiral isolates revealed aggregate alterations that permitted a diagnosis of interstitial nephritis. This characteristic interstitial nephritis was not observed in the two animals from which

*Leptospira biflexa* strains were isolated. Lesions encountered in these two animals were scanty and consisted merely of hyaline casts in the collecting tubules plus limited deposits of material staining positively with periodic acid Schiff stain (PAS) around the glomerular tufts.

The diagnosis of interstitial nephritis was characterized by inflammatory lymphoid infiltration. This varied in intensity from slight to severe (see Plate 1). In all of the animals this lesion was accompanied by deposits of PAS positive material around the glomerular tufts and appeared more pronounced in the vicinity of the convoluted tubules. In animals whose inflammatory infiltration appeared more intense, these deposits formed thick layers around the wall of the Bowman's capsules and convoluted tubules. Interstitial nephritis appeared intense in two animals, moderate in five, and only slight in four others.

Warthin-Starry silver staining revealed the presence of leptospire in four of the 15 culture-positive animals. Two of these, from which *Bataviae* group strains were isolated, showed an abundance of leptos-

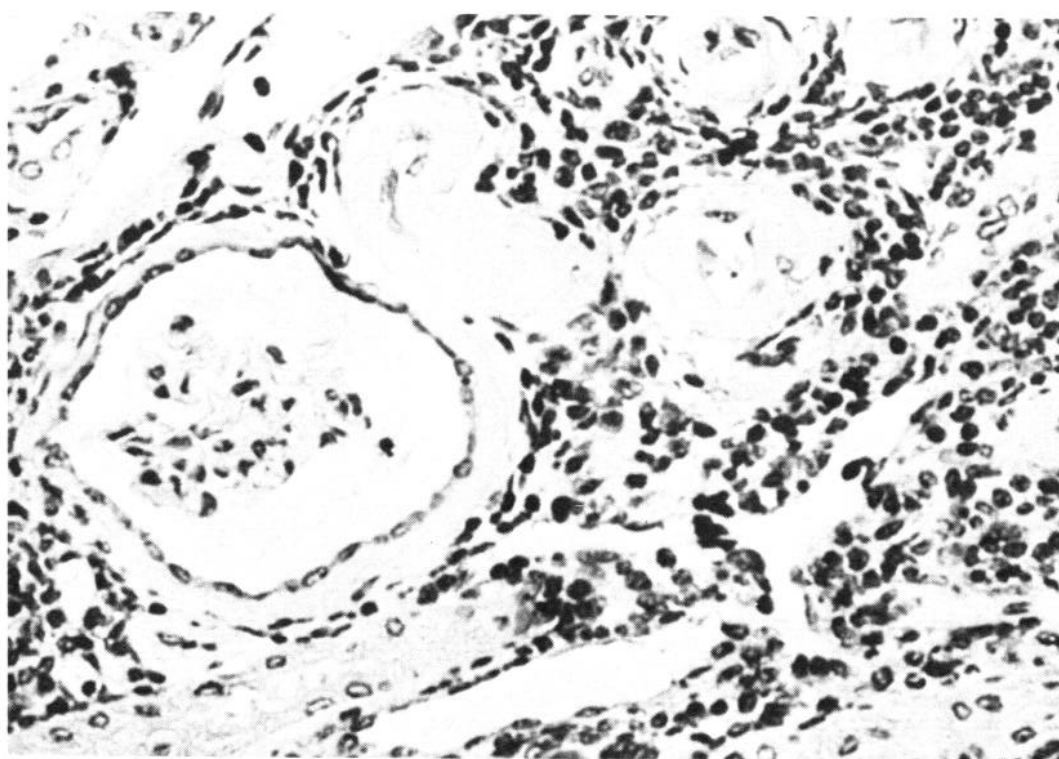


Plate 1—An armadillo kidney section showing marked thickening of the Bowman's capsule (at left) and lymphoid interstitial infiltration (at right). Stained with hematoxylin-eosin, x 400.

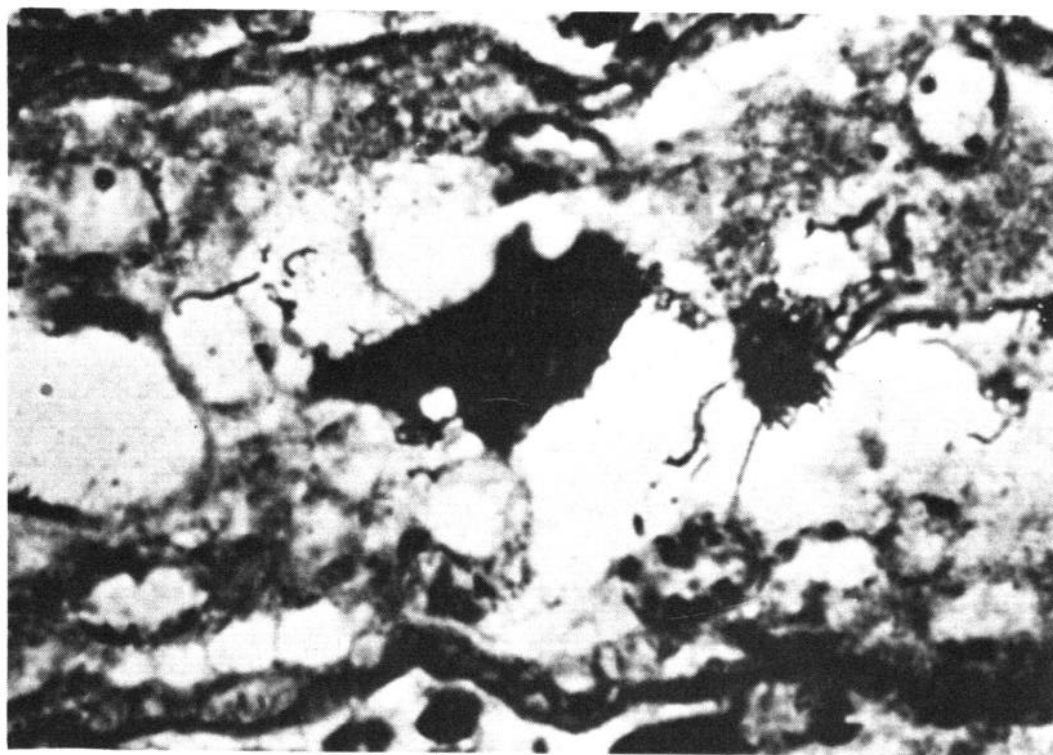


Plate 2—Armadillo kidney tissue treated with Warthin-Starry silver stain, showing an abundance of leptospire within a convoluted tubule, x 1200.

pires (see Plate 2). Of the other two positively stained kidneys, one came from the armadillo in which serotype *hardjo* was

isolated, and the other came from one of the animals yielding an isolate of *Leptospira biflexa*.

No evidence of interstitial nephritis was encountered in 60 kidneys from armadillos yielding no leptospiral isolates. However, lesions of minor pathological significance were observed in some of these animals. These lesions consisted mainly of hyaline casts and small areas of polymorphonuclear infiltration below the epithelium of the renal pelvis.

No histopathologic differences were observed with respect to differences in either the animals' weight and sex or the infecting leptospiral strain.

### Discussion

The findings in this study provide further evidence that a high percentage of armadillos in Argentina are infected with multiple leptospiral serotypes. A total of 16.8 per cent of the animals examined were found to have leptospires in their kidneys. Approximately 47 per cent of the sera from the captured animals showed predominant leptospiral agglutinins to serotypes of the Hebdomadis, Bataviae, Canicola, and Pomona sero-groups. This latter percentage was based upon our criterion that 50 per cent agglutination at a titer of 1:50 or more constituted a positive reaction. In this regard, it should be noted that serologic rates alone are not always valid indicators of the prevalence of infections in wildlife. For instance, when subjected to the MA test, sera from some animals may yield negative results for the same serotype with which the animals are actually infected.

The discovery of a high percentage of armadillos with Hebdomadis serogroup agglutinins in this study, similar to the findings reported by Carillo et al. (3), was interesting in view of that serogroup's close ecological relationship to cattle. Reactor rates among Argentine cattle to Hebdomadis group antigens are known to be high, and six isolations of serotype *hardjo* have been made from bovine kidneys (5). The present study, employing improved culture meth-

ods, isolated serotype *hardjo* from one armadillo captured on a cattle farm. It is not possible at this time to assess the epidemiologic importance of this one isolation in relation to bovine leptospirosis; but the occurrence does represent the first known isolation of *hardjo* from this animal species, and shows the armadillo to be a host susceptible to infection with this serotype.

The significance of the isolation from armadillo renal tissues of two apparently saprophytic leptospiral strains having characteristics comparable to *Leptospira biflexa* is unknown. Their appearance as possible contaminants is unlikely, since the kidneys were aseptically removed from the animals immediately before culture and the BA-P80 medium in which they grew had been prepared with distilled water and autoclaved. Furthermore, the Warthin-Starry silver staining technique detected leptospires in kidney sections from one of the two animals involved. Similar leptospires have previously been isolated from the frog by Diesch et al. (12) and from kidneys of apparently normal horses by Myers (13).

Histopathologic examination of kidneys from 13 of the culture-positive armadillos revealed evidence of renal lesions varying from slight to severe in 11 of the animals. The histopathologic appearance of the two armadillos from which the *Leptospira biflexa* strains were isolated was normal. No marked pathological differences were observed between the armadillos infected with Bataviae group strains, those yielding Canicola group strains, and the single animal infected with serotype *hardjo*. The most significant lesions—in order of intensity and frequency—were chronic interstitial nephritis, thickening of the Bowman's capsule, congestion of the glomerular tufts, and necrotic degeneration of the renal tubules. The interstitial nephritis appeared intense in two of the culture-positive armadillos examined, moderate in five others, and only slight in the remaining four.

According to Turner (14) and Smith et al. (15), a suitable carrier-host is one which is abundant, is easily infected, excretes leptospire, and still remains relatively unaffected by the infection. In this vein, the efficiency of a carrier may be estimated by finding the ratio of the culture isolation rate to the positive serology rate. If the serology rate greatly exceeds the culture isolation rate, it would appear that infec-

tions are not persistent and that no leptospire are excreted for long periods of time. Using this criterion, the present study's combined histopathologic findings of chronic nephritis and high rates of infection, based upon both serology and cultural isolations, suggest that the armadillo is an important natural reservoir-host for pathogenic leptospirae.

### SUMMARY

A serologic, bacteriologic, and histopathologic examination for leptospire was carried out on 89 armadillos (*Chaetophractus villosus*) from Argentina. Forty-seven per cent of the serum samples yielded positive results when tested by microscopic-agglutination. Predominant agglutination reactions were to the Hebdomadis and Bataviae serogroups.

A total of 15 *Leptospira* isolations (from 16.8 per cent of the animals tested) were obtained from kidney tissue. Nine of the isolates were identified as belonging to the Bataviae group serotypes *argentiniensis*, *paidjan*, or *bataviae*; three other isolates proved to be the Canicola group serotype *canicola*; two others were *Leptospira biflexa* strains; and the last isolate was found to be serotype *hardjo* of the

Hebdomadis group. The latter finding represents the first isolation of serotype *hardjo* from this animal species.

Histopathologic examination of kidneys from 11 of the animals yielding pathogenic leptospire permitted a diagnosis of interstitial nephritis. This interstitial nephritis, presenting the characteristic picture of lymphoid infiltration, appeared intense in two animals, moderate in five others, and only slight in the remaining four.

These histopathologic findings of chronic nephritis, combined with the high positive serologic and cultural isolation rates, suggest that the armadillo is an important natural reservoir-host for pathogenic leptospirae.

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