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SURVEY OF WILD MAMMALS IN A CHESAPEAKE BAY AREA FOR SELECTED ZOONOSES

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Abstract: Studies were conducted on 104 striped skunks, 97 raccoons, 64 opossums, 10 woodchucks, 5 feral cats, and 1 muskrat. Animals were trapped on an undeveloped forest and swamp area in Eastern Maryland. Leptospiral agglutinins were demonstrated in 62% of the skunks, 15% of opossums and 50% of the raccoons. Leptospiral serogroup members isolated from hosts were as follows: skunks — *icterohaemorrhagiae, grippotyphosa, ballum,* and *autumnalis;* opossum — *icterohaemorrhagiae* and *ballum;* raccoon — *icterohaemorrhagiae. Listeria monocytogenes* was isolated from one raccoon. Significant complement-fixing antibodies for Rocky Mountain Spotted Fever group of rickettsia were demonstrated in 15% of 102 skunks, 18% of 94 raccoons, 40% of 10 woodchucks and in only 1 of 54 opossums. Isolates of a pox virus which appears to be a new type were obtained from upper respiratory tissues of two raccoons. Significant antibody titers for the isolated pox virus were demonstrated in 23% of 92 raccoon sera. Two isolates serologically related to infectious canine hepatitis (ICH) virus were isolated from pooled liver and spleen tissues of two skunks. Sera from 59 of 94 skunks had neutralizing antibody to the ICH isolate. Examinations of sera for antibody to influenza viruses A and B were negative. Complement-fixing antibodies for toxoplasmosis were demonstrated in 10 of 80 raccoons, and 3 of 7 woodchucks, but not in other species. Three types of microfilariae were seen in bloods of 29 of 104 skunks, 3 of 97 raccoons, and 1 of 61 opossums. During dissection of animals the guinea worms *Dracunculus fuelleborni* and *Dracunculus insignis* were found in three opossums and one raccoon respectively. Lung flukes *(Paragonimus kellicoti,* syn. *P. rudis)* were found in three skunks.

INTRODUCTION

Surveys for selected zoonoses were conducted on small wild mammals, excluding rodents, located in an undeveloped forest and swamp area of Aberdeen Proving Grounds, Maryland. Diseases selected for study were within the scope of concurrent research projects or study facilities of the Division of Veterinary Medicine, Walter Reed Army Institute of Research (WRAIR). These included leptospirosis, listeriosis, rickettsiosis, viral infections, toxoplasmosis, and filariasis.

Findings of this extensive study are summarized in this report.

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MATERIALS AND METHODS

Source of Animals

Animals were trapped alive in an area comprising 35,000 acres, lying on the coastal plain adjacent to upper Chesapeake Bay near Aberdeen, Maryland. This area is composed of many forests and grasslands of irregular shapes. Because of this unusual interspersion of habitat and abundant small wet spots, some wildlife species such as deer, rabbits, raccoons, opossums, and skunks are unusually abundant. During the period May 1961 to May 1962, 281 captured animals were brought to WRAIR for study. The species and numbers collected were as follows: striped skunk *(Mephitis mephitis)* — 104, raccoon *(Procyon lotor)* — 97, opossum *(Didelphys marsupialis virginiana)* — 64, woodchuck *(Marmota monax)* — 10, muskrat *(Ondatra zibethica)* — 1, and feral house cat *(Felis domestica)* — 5.

Collection of Specimens

Animals were narcotized and killed by exposing them to a combination of chloroform and dry ice. Heart's blood samples were obtained for serological tests and for examinations for microfilariae. Heparin was used as an anticoagulant for the latter purpose. Sera were separated from clotted bloods and were stored at —70C. Animals were dissected aseptically and sections of brain, liver, spleen, lung, upper respiratory tract, and intestines were separately placed in sterile screw cap bottles, then rapidly frozen in an alcohol dry-ice bath and stored in a —70C freezer. At time of necropsy, portions of kidney tissues, and urine (obtained by bladder tap) were processed for cultivation of leptospiras. Also tissue suspensions in tryptose phosphate broth were prepared and swabs of vaginal mucosa were made for cultural tests for listeria.

Leptospirosis Examinations

Technics for cultural and serological tests are described in detail elsewhere.[1] Generally, kidneys were triturated by use of a mortar and pestle and suspended in nine volumes of sterile phosphate-buffered, physiological salt solution (pH 7.2). One portion of the suspension was diluted further ten-fold. Each suspension was cultured in four tubes of Fletcher's medium, using minimal inoculum, ca. 0.05 ml per 5 ml of medium. Undiluted urine and a one in ten dilution thereof were similarly cultured.

Sera were examined for leptospiral agglutinins by the microscopic-agglutination technic with live antigens from 18 different serotype strains.[9] Isolated leptospiras were identified on the basis of their cross-agglutination reactions with an extensive battery of serotype antisera.[1,9] Agglutinin-adsorption tests on one of the isolates were done according to previously described methods.[2,9]

Listeria Examinations

Brain suspensions, pooled liver and spleen suspensions and vaginal swabs were inoculated into tubes of tryptose-phosphate broth which in turn were subcultured after 18 hours incubation at 37C into Modified McBride's broth and agar media.[10] Cultures were examined and identified according to described procedures.[11,23]

Examinations for Rickettsia

Sera were tested for presence of complement-fixing (CF) antibodies for the Rocky Mountain Spotted Fever (RMSF) group of rickettsia in cooperation with the Department of Rickettsial Diseases (WRAIR) according to described procedures.[5]

Cultural Examinations for Viruses

Specific cultural procedures will be presented separately (McConnell, S. J. et al., to be published). Generally stored tissues were rapidly thawed, triturated, and suspended in a phenol red broth lactalbumin hydrolysate medium containing antibiotics. The suspensions were centrifuged at low speed to remove particulate matter. Supernatant fluids of same tissue suspensions from three to five animals were pooled and inoculated respectively into cultures of kidney tissues of pig, rabbit, dog, monkey, and hamster. Exceptions were suspensions of liver and

spleen from each animal which were pooled and cultured. At least three blind cultural passages were made including the use of the Dulbecco placquing technic for plaque formation before a test specimen was considered to be negative. Preparations from the upper respiratory tract samples were inoculated into embryonic rabbit lung tissue cultures which were sequentially passed into the same medium and monkey kidney cell cultures. The final blind passage was tested for the presence of hemadsorbing agents.

Serological Tests for Viral Antibodies

Conventional Tests[19] were used to determine the presence in sera of hemagglutination - inhibition (HAI) antibodies against influenza virus types A (PR8), A1 (Hawaii/303/56), A2 (Jap/305/57), and B (Va/303/56 and Lee). Antigens for this test were prepared in embryonated chicken eggs. Type "O" human red blood cells were used.

HAI tests for pox virus antibodies in raccoon sera were done with cardiolipin sensitive chicken erythrocytes. Strains used as antigens were an isolated raccoon pox virus (WR pox R6-10), vaccinia virus (CVI) and monkey pox virus (WR 7-61). The antigens were prepared from infected chorioallantoic membranes of chicken embryos according to described procedures.[7]

All skunk sera were tested for neutralizing antibodies from a skunk adenovirus isolate by the use of previously described methods.[4] A select number of skunk sera were also similarly tested with a reference strain of infectious canine hepatitis (ICH) virus (Cornell) and the related Toronto A 26/61 canine adenovirus.

Toxoplasma Tests

Sera were tested for toxoplasma antibodies by means of a complement fixation technic.[15] Antigen for this purpose was an extract of sonically-treated *Toxoplasma* cells obtained from peritoneal exudate of infected laboratory mice (A. Warner, unpublished data, WRAIR). Appropriate positive and negative controls were used for each test run.

Examination for Microfilariae

Heparinized blood was examined with the use of the acridine orange vital staining technic.[22]

RESULTS AND DISCUSSION

Leptospirosis

Leptospiras were isolated from 26 to 104 skunks, 3 of 64 opossums, and 1 of 97 raccoons but not from 10 woodchucks, 5 feral cats and 1 muskrat. All but 9 of the isolates were identified. The unidentified strains were inadvertently lost as a result of a laboratory accident. The 21 identified strains comprised members of the *icterohaemorrhagiae, grippotyphosa, ballum,* and *autumnalis* groups. The distribution of isolates among hosts is shown in Table 1. The high infectivity rate in skunks is similar to other findings but differed with regard to the relatively high prevalence of infections with *icterohaemorrhagiae*.[20,21] With one exception, the serogroup members found in the three species of mammals had previously been reported in other states.[20] The exceptional finding which was reported separately was *icterohaemorrhagiae* in an opossum.[9]

The results of microscopic agglutination tests for leptospiral antibodies are shown in Table 2. Titers ranged from 1:25 to 1:400. As would be expected, the percentages of seropositives were greater than those of carriers. The high prevalence of antibodies in raccoons was particularly high in contrast to the paucity of isolations. Four of 26 skunks that were leptospiral carriers had no detectible antibodies. Similar observations in skunks and various other animals have been made by others.[3,21] Four skunks had agglutinins for *grippotyphosa* although *icterohaemorrhagiae* was isolated. Such findings are not unusual and may reflect simultaneous or repeated infections with diverse serotypes.[21] Other serological findings were consistent with culture typing results. Serological test results also provided presumptive evidence of infection with serological types not recovered in culture, e.g., *pomona* in skunks, *autumnalis* in raccoons, *grippotyphosa* in opossums, etc. It was apparent that the site of study was an enzootic area of leptospirosis.

TABLE 1. Leptospiras isolated from wild mammals in Maryland.

Species*	No. Examined	Positives No. (%)	Distribution by Serogroup
Skunk	104	26 (25.0)	*icterohaemorrhagiae* — 12 *grippotyphosa* — 4 *ballum* — 1 *autumnalis* — 1 not typed — 8
Opossum	64	3 (4.7)	*icterohaemorrhagiae* — 1 *ballum* — 1 not typed — 1
Raccoon	97	1 (1.0)	*icterohaemorrhagiae* — 1

*No leptospiras isolated from 10 woodchucks, 5 feral cats and 1 muskrat.

TABLE 2. Prevalence of leptospiral agglutinins in sera of wild mammals in Maryland.

Species*	No. Tested	Positives No. (%)	Distribution of Predominant Reactions
Skunk	101	63 (62.4)	*grippotyphosa*—22, *icterohaemorrhagiae*—10, *autumnalis*—7, *ballum*—2, *djasiman*—2, *javanica*—1, *andamana*—2, *multiple*—17
Opossum	60	9 (15.0)	*grippotyphosa*—3, *autumnalis*—2, *ballum*—1, *icterohaemorrhagiae*—1, *andamana*—1, *multiple*—1
Raccoon	94	47 (50.0)	*autumnalis*—24, *icterohaemorrhagiae*—10, *pomona*—2, *grippotyphosa*—2, *canicola*—2, *borincana*—1, *javanica*—1, *australis*—1, *multiple*—4

* No agglutinins found in sera of 10 woodchucks, 5 feral cats, and 1 muskrat.

Listeriosis

Listeria monocytogenes was isolated from the pooled suspensions of liver and spleen from one adult raccoon only. No gross lesions were seen in tissues from this raccoon. The culture was serologically identified as type 4b. Gifford and Jungherr[10] reported the isolation of *L. monocytogenes* from a wild raccoon with a fatal septicemic infection. So far as we know inapparent listeriosis in a raccoon has not heretofore been reported. Possibly the isolation was made during an early phase of infection.

RMSF Group of Rickettsiae

Part of the serological findings were previously presented.[5] A summary of complete test findings is shown in Table 3. Significant antibody titers were found in 15 of 102 skunks, 17 of 94 raccoons, 1 of 54 opossums, and 4 of 10 woodchucks. CF titers ranged from 1:5 to 1:320. The findings were indicative of the enzootic occurrence of RMSF rickettsiae in the study area. The dog tick, *Dermacenter variabilis,* an important vector for RMSF was commonly seen on trapped animals.

Raccoon Pox

Only four viral agents were isolated in the extensive series of cultural attempts, two pox viruses and two adenoviruses.

A poxvirus isolate was obtained from preparations of pooled upper respiratory tissues of five raccoons (R 6-10). This preparation produced cytopathogenic effects (CPE) in monkey kidney tissue

TABLE 3. Prevalence of Rocky Mountain spotted fever complement-fixing antibodies in sera of wild mammals in Maryland.

Species	No. Tested	Positives No. (%)	Distribution of Positives by Titer* 5	10-20	40-80	160-320
Skunk	102	15 (14.7)	6	8	1	0
Raccoon	94	17 (18.1)	6	3	6	2
Oposum	54	1 (1.8)	0	0	1	0
Woodchuck	10	4 (40.0)	3	0	1	0

* Expressed as reciprocals.

cultures (MKTC). The CPE were seen on the 11th day of incubation and were characterized by rounding and granular appearance of cells. Subsequently the individual tissue suspensions which made up the R 6-10 pool were separately cultured. Pox virus was recovered from samples from two raccoons, either by cultivation on MKTC or in chicken embryos or on both media. When inoculated on the chorioallantoic membrane (CAM) of 12-day embryonated hens' eggs, the raccoon pox produced many small discrete, embedded pocks. Its identity as a member of the pox virus group was affirmed by a variety of standard tests, including its ability to agglutinate cardiolipin sensitive chicken erythrocytes.[12] Hemagglutination was specifically inhibited by anti-vaccinia rabbit serum. The titer of the anti-vaccinia serum with a homologous CAM antigen was 1:640; it was 1:320 with a raccoon pox CAM antigen.

Results of HAI tests on 92 raccoon sera conducted with raccoon pox, vaccinia, and monkey pox antigens are shown in Table 4. Twenty-two sera contained significant HAI antibodies for pox isolates ranging from 1:80 to 1:2560. Three of 22 positive sera also had demonstrable titers against vaccinia; no reactions were elicited with the monkey pox antigen. Both raccoons from which pox viruses were isolated had serum HAI antibody titers; one had titers of 1:2560 and 1:1280 respectively against the raccoon pox and vaccinia antigens, the other had a titer of 1:320 only with the raccoon pox antigen. No gross lesions were seen in tissues of either raccoon at necropsy.

In view of the negative serologic findings in raccoon sera with monkey pox antigens, additional tests were done to determine if reciprocal reaction occurred between the two pox viruses. Twenty rhesus monkey sera, known to contain HAI antibodies for monkey pox, did not inhibit the hemagglutinating activity of raccoon pox antigen.

The cross-reactivity of the raccoon pox virus with vaccinia coupled with its hemagglutinating properties would justify

TABLE 4. Prevalence of pox virus hemagglutination inhibition antibodies in sera of raccoons trapped in Maryland.

Antigens	No. Tested	Positives No. (%)	Distribution of Positives by Titer* 80	160	320	640	1280	2560
Raccon pox (R 6-10)	92	22 (23.8)	6	8	4	3	0	1
Vaccinia (CV-1)	92	3 (3.3)	0	0	0	2	1	0
Monkey pox (WR 7-61)	92	0 (0)	0	0	0	0	0	0

* Expressed as reciprocals.

its inclusion in the variola-vaccinia subgroup of pox viruses. However in contrast to other known members of this pox virus group, the raccoon pox was serologically unique in its inability to cross-react with the monkey pox agent. The serological and immunological relationships of raccoon pox virus and other pox viruses require additional study. It was apparent from serological findings that this newly disclosed pox virus was highly prevalent in the raccoon population of the study area. Its prevalence in other wildlife hosts and its potential veterinary and public health significance should be studied further.

Adenovirus

Viruses were recovered from pooled suspensions of liver and spleen tissues from two skunks. The agents produced CPE in dog kidney tissue cultures. The viruses were identified to be adenoviruses on the basis of physical, chemical, cultural, and serological properties. The detailed characterization studies which established the identity of the two viruses will be presented separately (McConnel et al., to be published). The CPE produced by either agent in dog kidney tissue culture were inhibited specifically by anti-infectious canine hepatitis sera. The possible orign of the two agents from dog kidney cell cultures was ruled out by the reisolation of the viruses from the original samples. Furthermore, sera from 59 of 94 skunks had neutralizing antibody to one of the isolates chosen as a reference strain (SK 73) when screened at a 1:4 dilution. Neither skunk which provided an isolate had detectible antibody. One had pin point hemorrhages on the lung; the second culturally-positive skunk had no apparent lesions. Presumably the isolates were obtained during the early phase of infection.

Eight of 59 seropositive sera were randomly chosen for additional neutralization tests with the use of antigens from reference strains of ICH (Cornell), the Toronto A 26/61 canine adenovirus as well as SK-73. Results of tests are shown in Table 5. Titers ranged from 1:20 to 1:1280 or greater. In comparative serological reactions, the skunk isolate was indistinguishable from ICH virus. ICH virus has not previously been reported in skunks. Except for presented findings in skunks, the known occurrence of ICH in dogs and foxes and a recent report of ICH in zoo bears, there is little information about ICH in wildlife.[6,14]

Influenza

No antibodies for influenza types A and B were found in wildlife sera.

Toxoplasmosis

Sera were tested at a singel dilution level of 1:8. Ten (12.5%) of 80 raccoons and 3 of 7 woodchucks were positive. No antibodies were found in sera from 42 opossums, 34 skunks, and 5 feral cats. The disclosure of toxoplasma antibodies in wildlife was consistent with other findings.[13,21]

TABLE 5. Neutralizing antibody titers of select skunk sera for adenoviruses, SK-73, ICH, and A 26/61.

Sera	Neutralizing Antibody Titer* With Virus SK-73	ICH	A/26/61
Skunks 40, 75, 100, 102	$\geq$ 1280	$\geq$ 1280	$\geq$ 1280
Skunk 3	1280	$\geq$ 1280	320
Skunk 8	$\geq$ 1280	$\geq$ 1280	80
Skunk 86	320	320	20
Skunk 48	20	20	$<$ 20
ICH antiserum	320	320	80
A 26/61 antiserum	20	20	320

* Titers expressed as reciprocals. Neutralizing tests were done on dog kidney tissue cultures. The log of tissue culture ID_{50} of SK 73, ICH, and A 26/61 viruses were 2.3, 1.8, and 2.5 respectively.

Helminths

Microfilariae were found in bloods of 29 (27.9%) of 104 skunks, 3 (3.1%) of 97 raccoons and 1 (1.6%) of 61 opossums. No microfilariae were found in ten woodchucks, six feral cats, and one muskrat. Three types of microfilariae could be differentiated. A long unsheathed form measuring 250-275 by 4 μ was found in 15 skunks and one raccoon. This first type fits the description of *Dipetalonema interstitium* found in a grey squirrel in Maryland.[16] Similar microfilariae have been found in skunks and raccoons of Maryland.[17] A second shorter unsheathed form, 175-225 μ long and 3-4 μ wide was found in 12 skunks and two raccoons. The unsheathed microfilariae resemble *Dipetalohema mephitis* which was found in Canadian skunks and described by Webster and Beauregard.[25]

Both unsheathed forms were usually present in concentrations of 100 or more per drop of blood. A third short sheathed form, measuring 140-165 μ long by 4-5 μ wide was found in sparse concentration (e.g., 1 to 4 filariae per drop of blood) in 4 skunks and 1 opossum. It resembled previously described microfilariae found in Maryland wild mammals.[8,17]

During necropsy, guinea worms identified to be *Dracunculus fuelleborni,* were found in subcutaneous tissues of three opossums. Another guinea worm, *Dracunculus insignis,* was found in a raccoon. Lung flukes *(Paragonimus kellicoti,* syn. *P. rudis)* were found in three skunks.

The considerable number of new findings in this study point up the wealth of information that remains to be uncovered by the interested investigators of infectious diseases of wildlife.

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