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AEGYPTIANELLA RANARUM SP. N. (RICKETTSIALES, ANAPLASMATACEAE): ULTRASTRUCTURE AND PREVALENCE IN FROGS FROM ONTARIO

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ABSTRACT: *Aegyptianella ranarum* sp. n. (Rickettsiales, Anaplasmataceae) was recorded from bullfrogs (*Rana catesbeiana* Shaw), green frogs (*Rana clamitans* Latreille) and mink frogs (*Rana septentrionalis* Baird) from five sites in southern Ontario. The rickettsia occurs within membrane-bound vacuoles in the cytoplasm of erythrocytes with up to 120 organisms in mature inclusions. The pattern of replication of *A. ranarum* in host erythrocytes and its prevalence over a 3-yr period in frogs from Algonquin Park, Ontario are discussed.

INTRODUCTION

The erythrocytes of amphibians and reptiles are infected commonly with a variety of protozoan parasites. Other intraerythrocytic organisms, too small to be characterized by light microscopy, have been suspected to be prokaryotes and were referred to, among other designations, as species of *Pirhemocyton*, *Cytamoeba* and *Toddia* (see review by Johnston, 1975).

Electron microscopic examination of the contents of spherical inclusions in the erythrocytes of green frogs (*Rana clamitans* Latreille) from Algonquin Park, Ontario revealed the presence of many closely spaced, rickettsia-like prokaryotes (Desser and Barta, 1984).

Further ultrastructural study of infected frogs from the same locality has provided novel information on the intraerythrocytic development of the rickettsial organisms and prompted the description of a new species in this report. Additional survey data indicate a wider host and geographical range for the organism.

MATERIALS AND METHODS

During the summers of 1983 to 1985, a survey of blood parasites was made from 550 bullfrogs (*Rana catesbeiana* Shaw), 282 green frogs (*Rana clamitans* Latreille), 384 mink frogs (*Rana septentrionalis* Baird), 90 wood frogs (*Rana sylvatica* Le Conte) and 82 American toads (*Bufo americanus* Holbrook) from a sphagnum bog and adjacent forest on the southwest shore of Lake Sasajewun, Algonquin Provincial Park, Ontario (lat. 45°35′N, long. 78°30′W). The amphibians were examined for ectoparasites and snout to vent and leg lengths were recorded. They were marked by toe-clipping to ensure that specimens were not re-examined if recaptured. Blood films were prepared by removing the tip of a digit and smearing blood from the cut face directly onto a slide. The blood films were fixed in methanol and stained with Giemsa's stain in phosphate buffer pH 7.2. The parasites were photographed on Kodak Panatomic X film in a Zeiss Universal 1 photomicroscope.

Ultrastructural observations were made on the intraerythrocytic inclusions in the following manner. Blood from an infected green frog was fixed in a suspension in 0.05 M PIPES (piperazine-N,N′-bis[2-ethane-sulfonic acid]) buffered 1.25% glutaraldehyde (pH 7.0, 300 mOsm) for 1 hr at room temperature, washed in 0.12 M PIPES buffer and post-fixed in 1.0% OsO_4 in 0.12 M PIPES. The blood cells were dehydrated in a graded ethanol series and embedded in Spurr's medium, according to the method of Hong and Barta (1986). Ultrathin sections were stained with uranyl acetate and lead citrate and examined using either a Zeiss E.M. 9A or a Philips 201C electron microscope operating at an accelerating voltage of 60 kV.

To determine whether the rickettsial organism occurred in frogs from other localities in

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the province, blood was examined from animals from several locations in southern Ontario. These sites included a roadside sphagnum bog near Winchester, a grass ditch near Corbyville, a sphagnum bog near Coboconk, a cattail swamp on the margin of Pigeon Lake and a shallow pond in the Dundas Valley Conservation Area (see Fig. 1).

RESULTS

Light microscopy

The inclusions were roughly spherical and measured from 3 to 11 μm in diameter. Small inclusions were often densely stained and appeared to contain several closely packed rickettsiae arranged in parallel (Fig. 3). The larger inclusions contained lighter staining, filamentous material (Figs. 2, 4). The inclusions were surrounded by a narrow, darkly stained band.

Electron microscopy

Small inclusions differed strikingly in appearance from the large ones. The smaller forms lay within a membrane-bound vacuole in the cytoplasm of the erythrocyte (Figs. 5, 7 inset) and contained a moderately dense, irregularly arranged granular matrix (Figs. 5, 7). Large spherical bodies of variable density and smaller patches of electron dense material were often seen in the smaller inclusions. Rod-shaped microorganisms, often arranged in parallel were embedded in the granular matrix of the small inclusions (Fig. 7).

The larger inclusions contained from 90–120 closely-spaced organisms, usually arranged in parallel, within a lucent matrix consisting of scattered granular material (Figs. 5, 10). The rickettsial nature of the organisms was revealed clearly at higher magnification. The organisms had a gram negative type of cell wall and were bound by a trilaminar membrane which was coated externally by dense fuzzy material. The outer membrane was separated by a narrow lucent zone, the periplasmic space, from the plasma membrane (Fig. 6). The interior of the organism consisted of dense, flocculent, peripherally arranged material and a lighter filamentous core, the nucleoid (Figs. 5–9). Rickettsiae in various stages of binary fission were often seen, especially in inclusions of intermediate size (Figs. 8, 9). Organisms in the larger inclusions measured 1–1.7 μm in length by 200–300 nm in diameter and appeared more dense than those in the smaller inclusions. Although rickettsiae were not observed in the process of entering erythrocytes, groups of free organisms were seen occasionally (Fig. 10).

Consideration of the above data prompted the following description of a new species of rickettsial organism in the Genus *Aegyptianella*.

Taxonomic summary

***Aegyptianella ranarum* sp. n.**

Hosts and locality: Bullfrog (*Rana catesbeiana*), green frog (*Rana clamitans*), mink frog (*Rana septentrionalis*), Algonquin Park, and several other locations in southern Ontario.

Description: In Giemsa-stained blood films, spherical intraerythrocytic inclusions 3–11 μm in diameter. Smaller inclusions contain densely-stained rods, arranged in parallel. Larger inclusions with dark stained boundary contain pink filamentous material.

The largest inclusions contain 90–120 rod-shaped organisms which measured 1–1.7 μm long by 200–300 nm in diameter. Organisms bound by two trilaminar membranes separated by a narrow periplasmic space; the interior consists of dense flocculent, peripherally arranged material and a lighter filamentous core region. Organisms divide by binary fission.

Specimens deposited: Blood films from green frogs infected with *A. ranarum* have been deposited in the Invertebrate Collection, National Museum of Canada, Ottawa, Canada (NMCIC(P) 1986-0033).

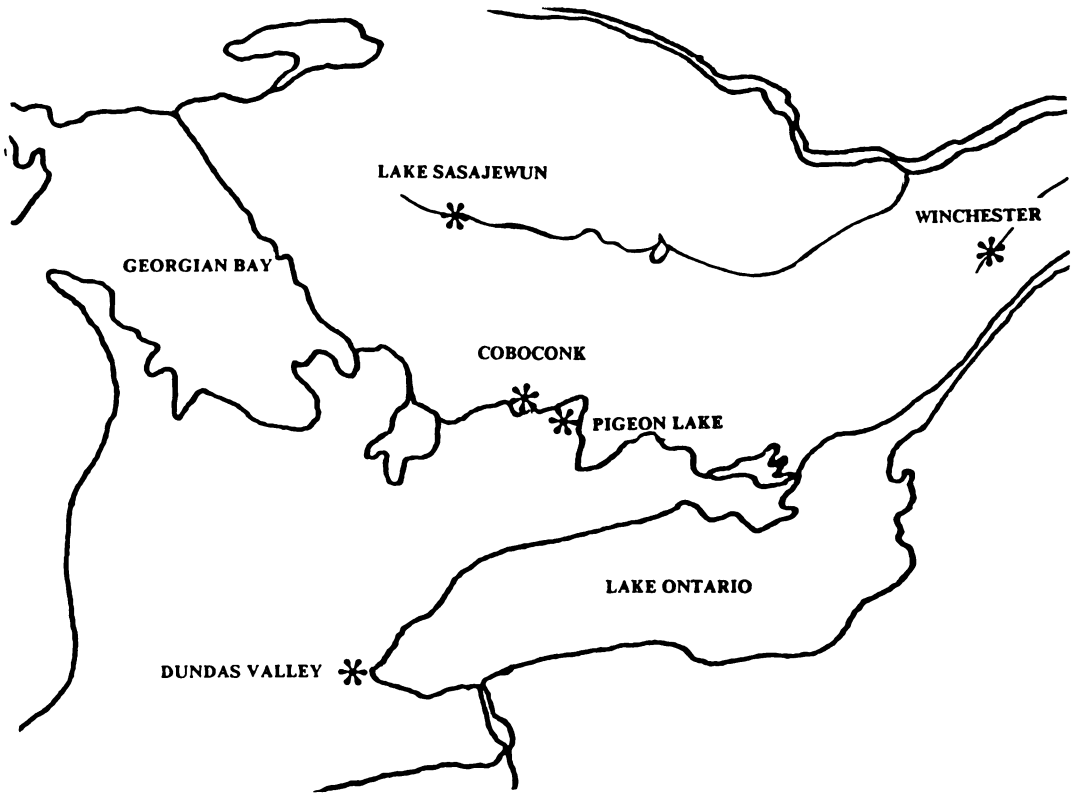

FIGURE 1. Distribution (asterisk) of *Aegyptianella ranarum* sp. n. in frogs from southern Ontario.

Comments

Of the genera in the Order Rickettsiales, only those of the family Anaplasmataceae are commonly intraerythrocytic parasites. Whereas species of *Anaplasma* infect large ruminants, those of *Aegyptianella* occur in the erythrocytes of birds and certain poikilotherms (Moulder, 1974). The intraerythrocytic inclusions of the latter species are segregated from the host cell cytoplasm within a membrane-bound vacuole. Individual organisms are morphologically similar to those of *Anaplasma* spp. i.e., are surrounded by a double trilaminar membrane (cell wall and plasma membrane) and contain dense aggregates of granular material embedded in an electron lucent substance (Moulder, 1974). Up to 26 rod-like bodies may be found in a single inclusion of *Aegyptian-*

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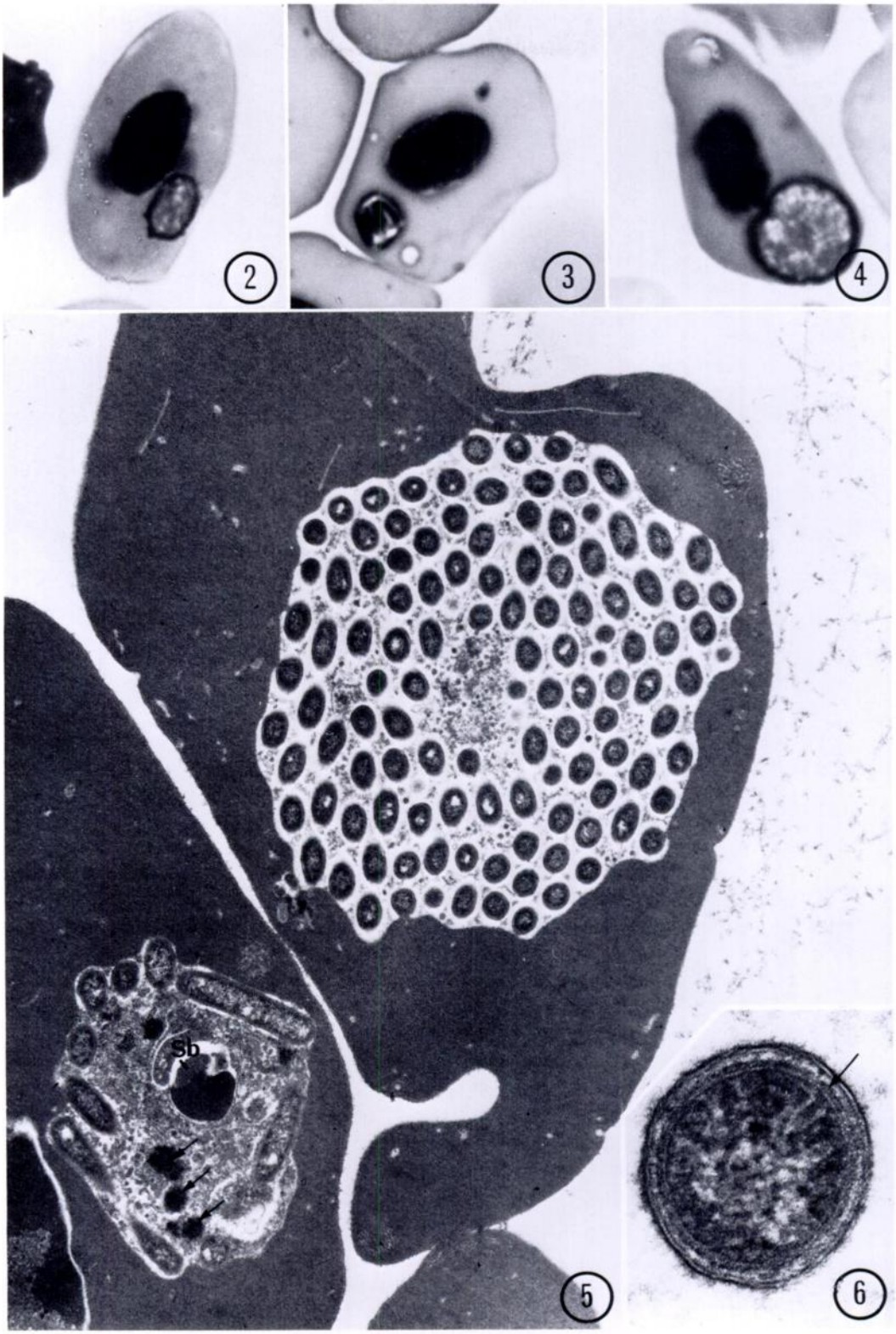
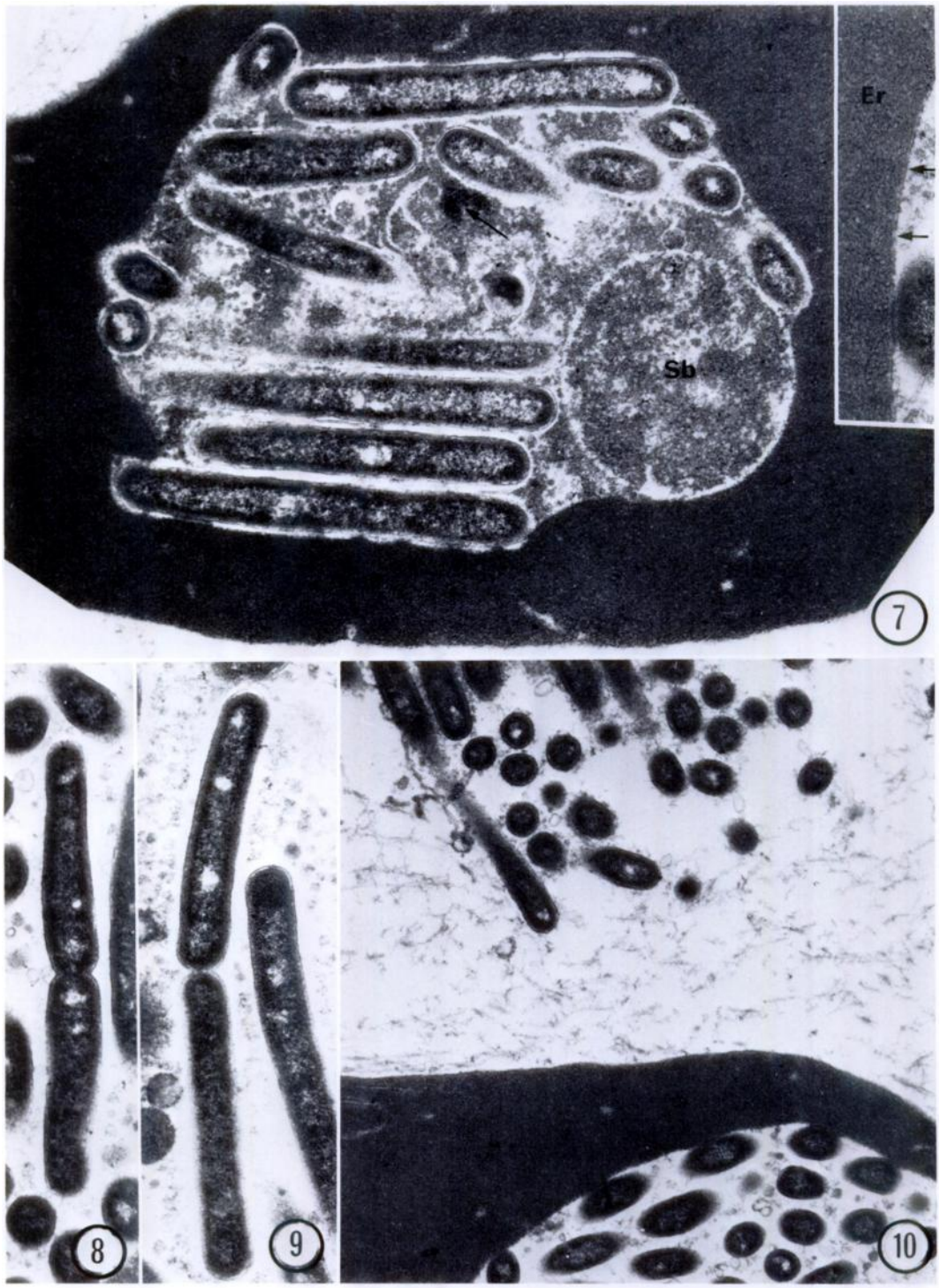
FIGURES 2–4. Photomicrographs of Giemsa-stained erythrocytes from *Rana clamitans* containing *Aegyptianella ranarum* sp. n. ×1,750. 2. Small inclusion with amorphous contents. 3. Small inclusion with "immature" rickettsiae arranged in parallel. 4. Large inclusion with filamentous contents.

FIGURES 5, 6. Electron micrographs illustrating stages of *Aegyptianella ranarum* sp. n. 5. Erythrocytes containing an "immature" inclusion (on the left) and a "mature" inclusion on the right. The latter contains about 115 organisms within a membrane-bound vacuole in the host cell cytoplasm. Note the dense spherical body (Sb) and the smaller dense patches (arrows) in the granular matrix of the "immature" inclusion. ×19,300. 6. Transversely sectioned organism from "mature" inclusion. The outer trilaminar layer (cell wall) is coated by a dense fuzzy material. A second, inner plasma membrane (arrow) surrounds the internal region which is composed of a dense peripheral zone and a lighter more filamentous core. ×174,000.





FIGURES 7–10. Electron micrographs illustrating stages of *Aegyptianella ranarum* sp. n. 7. "Immature" inclusion showing typical granular matrix. Note large spherical body (Sb), clump of dense material (arrow)

ella pullorum Carpano, the best known species of this genus (Gothe and Kreier, 1977).

Species of *Aegyptianella* are thought to infect poikilotherms also. Brumpt and Lavier (1935) observed spherical inclusions measuring 2.0–5.0 μm in erythrocytes from a turtle obtained at a Paris market. The authors considered that the large size of the inclusions (as compared to *A. pullorum*) as well as differences in staining, precluded including these parasites in the genus *Aegyptianella*. They erected a new genus and named the parasite *Tunetella emydis*. Based on the early description and drawings, Gothe and Kreier (1977) transferred this organism to the genus *Aegyptianella*. Although the intraerythrocytic inclusions of *A. ranarum* in frogs from Ontario are considerably larger than inclusions in birds and contain many more organisms, ultrastructural similarities between the parasites in birds and frogs indicate that they are species of the same genus. Interestingly, the intraerythrocytic inclusions of *A. ranarum* in frogs are larger than those of *A. pullorum* in birds in proportion to the differences in size of the host erythrocytes. The present description broadens the definition of the species *Aegyptianella* to accommodate intraerythrocytic inclusions in frogs containing up to 120 organisms.

Prevalence and geographical distribution of *A. ranarum* in southern Ontario

Data from the 1983–1985 surveys revealed the presence of *A. ranarum* in bullfrogs, green and mink frogs, but not in wood frogs or American toads (Table 1). The prevalence of infection varied considerably among the frog hosts from year to year. *Aegyptianella ranarum* was not observed in any of 75 bullfrogs during 1983, but occurred with 27% prevalence the following summer. Green frogs were infected in all 3 years. The lowest prevalence was recorded in mink frogs with 3.8% infected in 1984 and none in the other 2 yr.

The survey of frogs from other sites in southern Ontario revealed the presence of *A. ranarum* in green frogs from four of five localities sampled with a range in prevalence from 6.0 to 69.2% (Fig. 1, Table 1). The organism was not recorded from either bullfrogs or mink frogs, because these species were either scarce or difficult to capture at these sites.

DISCUSSION

The small intraerythrocytic inclusions observed in frogs in this study contained abundant granular material in which rickettsial organisms could be recognized. These "immature" rickettsiae appeared less dense than the "mature" forms in the large inclusions.

Despite considerable effort the mode of entry and early stages of development of rickettsiae are understood poorly (Hase, 1985). Following entry of *Rickettsia tsutsugamushi* (Hayashi) Ogata into its host cells, apparently by a process of facilitated phagocytosis (Moulder, 1985), there is a period during which the organisms are absent or difficult to detect before their reappearance and commencement of rapid multiplication. Hase (1985) postulated that at the initiation of infection, the rickettsial organism becomes disassembled and its free genome occurs in the cytoplasm of the host cell in the form of amorphous granular material. Daughter organisms are

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and rickettsial organisms. ×34,200. Inset. Boundary between erythrocyte (Er) and membrane-bound inclusion containing the parasites. Note the trilaminar vacuolar membrane (arrows). ×53,500. 8, 9. Longitudinally sectioned organisms undergoing binary fission. ×35,000. 10. Free rickettsiae adjacent to an erythrocyte containing a "mature" inclusion. ×24,100.

TABLE 1. Prevalence (%) of *Aegyptianella ranarum* sp. n. in frogs from southern Ontario.

Species of frog	Lake Sasajewun 1983	Lake Sasajewun 1984	Lake Sasajewun 1985	Winchester 1985	Corbyville 1985	Coboconk 1985	Pigeon Lake 1985	Dundas Valley 1985
Rana catesbeiana	0.0	27	11.5	—	—	—	—	0.0
	(75)*	(222)	(253)					(2)
Rana clamitans	3.5	23.5	27.3	6.0	0.0	23.5	69.2	17.6
	(57)	(87)	(148)	(50)	(6)	(17)	(13)	(15)
Rana septentrionalis	0.0	3.8	0.0	—	—	—	—	0.0
	(75)	(133)	(176)					(6)
Rana sylvatica	0.0	0.0	0.0	—	—	—	—	—
	(57)	(20)	(13)					
Bufo americanus	0.0	0.0	0.0	—	—	—	—	—
	(51)	(20)	(11)					

* Sample size.

assembled from the latter material. Possibly the small granular inclusions seen in the erythrocytes of frogs represent an early stage of assembly of *A. ranarum*. A second phase of assembly appeared to occur in the larger inclusions through replication of the organisms by binary fission, culminating in the formation of about 100 daughter cells.

Aegyptianella ranarum is ultrastructurally similar to other rickettsiae (Moulder, 1974), particularly the intraerythrocytic stages of *A. pullorum*, with which they share localization within a membrane-bound vacuole (Bird and Garnham, 1969; Castle and Christensen, 1985).

Although the observation of *A. ranarum* in frogs from Ontario represents the first record of this organism in Canada (Barta and Desser, 1984), they were recorded more than 80 yr ago in the United States. Stebbins (1904) described and illustrated similar inclusions in the erythrocytes of *R. catesbeiana* from Long Island, New York. He mistook these inclusions for haemogregarine gametocytes. Subsequently, Hegner (1921) observed these organisms in erythrocytes of *R. catesbeiana* and *R. clamitans* from the same locality in New York State. Hegner referred to the inclusions as *Cytamoeba bactifera* and speculated that this organism was a stage of a protozoan parasite and that "living within it either as a hyperparasite or in symbiosis is a bacillus named by Laveran *Bacillus krusei*." Similar intraerythrocytic inclusions containing bacilliform organisms were described also from frogs (by Laveran, 1899 and others), from African frogs (by Dutton et al., 1907) and from frogs of Madagascar (by Brygoo, 1963). Because of the limited resolution of the light microscope, the nature of the bacilliform organisms in the latter reports was unclear.

The available evidence suggests that *A. ranarum* is a cosmopolitan parasite of frogs which appears to exhibit specificity for species of *Rana* as evidenced by the records of the rickettsia in *Rana catesbeiana*, *R. clamitans* and *R. septentrionalis* in eastern North America, in *Rana esculenta* Linnaeus from Europe (Laveran, 1899) and in *Rana mascareniensis* Dumeril and Bibron from Africa and Madagascar (Dutton et al., 1907; Brygoo, 1963).

Arthropods, including fleas, lice, mites and ticks are known to serve as vectors of rickettsial diseases of homeothermic vertebrates (Moulder, 1974). Gothe (1967) demonstrated that *Aegyptianella pullorum* developed within the intestinal epithelium, hemocytes and salivary glands of *Argas walkerae* Kaiser and Hoogstraal

1969 and that the tick served as the vector of the rickettsia to fowl.

Preliminary evidence (Desser, unpubl. data) indicates that the intermediate host and vector of *A. ranarum* in Algonquin Park is the common amphibian-feeding leech, *Batracobdella picta* Verrill, in which the rickettsia undergoes prolific development.

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