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Source: Journal of Wildlife Diseases, 25(3) : 313-318

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-25.3.313

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*Journal of Wildlife Diseases*, 25(3), 1989, pp. 313–318
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# THE MORPHOLOGICAL FEATURES OF *AEGYPTIANELLA BACTERIFERA*: AN INTRAERYTHROCYTIC RICKETTSIA OF FROGS FROM CORSICA

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ABSTRACT: Study of ultrathin sections and freeze-fracture replicas of erythrocytes containing *Aegyptianella bacterifera* (Rickettsiales; Anaplasmataceae) revealed that the organisms develop within a membrane bound vacuole in the erythrocyte cytoplasm. The organisms divide by binary fission to produce about 12 rickettsiae in a mature inclusion. The organisms have gram-negative cell envelopes. The distribution of intramembranous particles within the outer and plasma membranes of *A. bacterifera* is similar to that of other rickettsiae and gram-negative bacteria such as *Escherichia coli*. The definition of the genus *Aegyptianella* should be broadened to include rickettsiae measuring up to 5 μm in length prior to division which develop within membrane bound inclusions in erythrocytes of their hosts.
*Key words:* Intraerythrocytic rickettsia, *Aegyptianella bacterifera*, ultrastructure, freeze-fracture, frogs, *Rana esculenta*, morphology.

## INTRODUCTION

Intraerythrocytic inclusions containing tiny organisms have been described from poikilotherms in several localities (reviewed by Johnston, 1975), but the determination of their affinities was hampered by their small size. In amphibians, intraerythrocytic inclusions containing elongate, densely stained organisms were first described from *Rana esculenta* and named *Cytamoeba bacterifera* by Labbé (1894). Similar inclusions were subsequently described from a variety of frogs from many localities. The rickettsial nature of the intraerythrocytic inclusions was first revealed in *Rana clamitans* from Ontario, Canada (Desser and Barta, 1984). Desser (1987) named the organism *Aegyptianella ranarum* and described its morphological features and prevalence. A recent survey of the blood parasites of frogs (*Rana esculenta*) from Corsica revealed intraerythrocytic inclusions containing *Aegyptianella bacterifera* (Barta et al., 1989).

In this study, *A. bacterifera* was examined by electron microscopy for the first time. Its affinities to other intraerythrocytic rickettsiae was confirmed. The membranes of this rickettsia, and the vacuole which encloses the organisms within the frog erythrocyte, were examined by freeze-fracture techniques. The taxonomic affinities of species of *Aegyptianella* in the blood of poikilotherms and the definition of the genus are discussed.

## MATERIALS AND METHODS

### Light microscopy

During early October 1986, specimens of *Rana esculenta* were collected along the banks of the Bravonne and Fium 'Orbo Rivers on the Island of Corsica (42°11'N, 09°32'E). Blood smears from these frogs were air-dried, fixed with methanol and stained with Giemsa's stain (1:20 in 0.1 M Sorensen's phosphate buffer, pH 7.2) for 45 min. Measurements were made using an ocular micrometer and are presented as a mean (SD) followed by sample size. Photomicrographs were taken with a Zeiss Universal 1 photomicroscope (Carl Zeiss Canada Ltd., 45 Valleybrook Drive, Don Mills, Ontario, Canada M3B 2S6) using Kodak Panatomic-X film (Kodak Canada Inc., 3500 Eglinton Avenue West, Toronto, Ontario, Canada M6M 1V3).

### Standard transmission electron microscopy preparation

Infected blood was fixed by allowing a drop to clot and then placing the clot directly into 2.5% (v/v) glutaraldehyde in 0.09 M Sorensen's phosphate buffer (pH 7.2) containing 0.02% (w/v) $CaCl_2$ (550 mOsm/kg $H_2O$) for 1 hr. The clot was washed in 0.2 M Sorensen's buffer containing 0.15 M sucrose and postfixed for 1 hr with 1% $OsO_4$ in 0.13 M Sorensen's buffer containing 0.8% (w/v) $K_3Fe(CN)_6$ and 0.15 M su-

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crose. Clots were then washed in distilled water and en bloc stained with 0.5% (w/v) aqueous uranyl acetate for 1 hr. The cells were dehydrated in a standard ethanol series, and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were stained using 2.0% (w/v) uranyl acetate in 50% (v/v) methanol followed by lead citrate (Reynolds, 1963).

### Freeze-fracture replica preparation

Blood clots were prepared for freeze-fracture by fixing the cells in the glutaraldehyde fixative as described above for 4 hr and washing with 0.2 M Sorensen's buffer. Specimens were infiltrated with 30% glycerol, mounted on gold stubs, and plunged into Freon 22 slush at −150 C for 10 sec, before transferring to liquid nitrogen for storage until fracturing. Specimens were fractured in a Balzer BAF 400 freeze-etch unit (Technical Marketing Associates Ltd., 6620 Kitimat Road, Unit 6, Mississauga, Ontario, Canada L5N 2B8) at −105 C with a vacuum of 1 × $10^{-6}$ Torr. Fractured surfaces were replicated by evaporating a 2 nm film of platinum at 45° and a 30 nm carbon layer at 90° using resistance evaporation guns. Replicas were immersed in absolute methanol, passed through a descending methanol series, rinsed in distilled water, and finally transferred to 6% sodium hypochlorite (bleach) overnight to digest away the adherent cells. Replicas were then passed through descending grades of bleach, rinsed four times with distilled water, and finally mounted on ultra high transmission grids.

### Electron microscopy

Ultrathin sections and freeze-fracture replicas were examined using a Philips 201C transmission electron microscope (Philips Electronics Industries Ltd., 601 Milner Avenue, Scarborough, Ontario, Canada M1B 1M8) operated at an accelerating voltage of 60 kV.

## RESULTS

### Prevalence

Forty-five percent (9 of 20) of the frogs captured in the Bravonne River area and 76% (50 of 66) of those animals examined along the banks of the Fium 'Orbo River were infected.

### Light microscopy

The organisms lay within a translucent, spherical to elliptical vacuole in the polar region of erythrocytes. The vacuoles contained from one to about 12 dense, elongate organisms which measured 2.3 (0.5) to 4.9 (0.6) ($n = 25$) by about 0.5 $\mu$m (Figs. 1–4). Even vacuoles containing one to a few organisms were well developed (Fig. 1).

### Electron microscopy of thin sections

The elongate organisms were loosely arranged in a membrane bound vacuole which contained a variety of small vesicles and other inclusions. These consisted of tiny dense particles and spherical bodies of varying size and density (Fig. 4). The rickettsial nature of the organisms was revealed at higher magnification (Fig. 5). They were bound by a gram-negative-like cell wall consisting of an outer membrane overlying a fine dense layer. This was separated by a narrow translucent zone, the periplasmic space, from the underlying plasma membrane (Fig. 5). The interior of the organisms consisted of mixed dense, flocculent and lighter filamentous materials. The denser material occurred predominantly at the periphery of the cells. Evidence of binary fission was seen in longitudinally sectioned organisms (Fig. 6).

### Electron microscopy of freeze-fractured specimens

The organisms lay within a membrane bound vacuole in the erythrocyte cytoplasm (Fig. 7). In addition to the rickettsiae, the vacuoles contained numerous membranous vesicles which corresponded in size to the spherical vesicles observed in thin sectioned material. The membranes forming these vesicles were devoid of intramembranous particles (IMP's) (Fig. 7). The organisms fractured predominantly between the lipid bilayers of the outer membrane or plasma membrane. The relative density and arrangement of the IMP's differed considerably between these two membranes. The E-face of the plasma membrane had numerous, randomly arranged IMP's (Figs. 7, 8) whereas the P-face of the same membrane had few IMP's (Fig. 9). The outer membrane had more densely packed IMP's. The E-face of the outer

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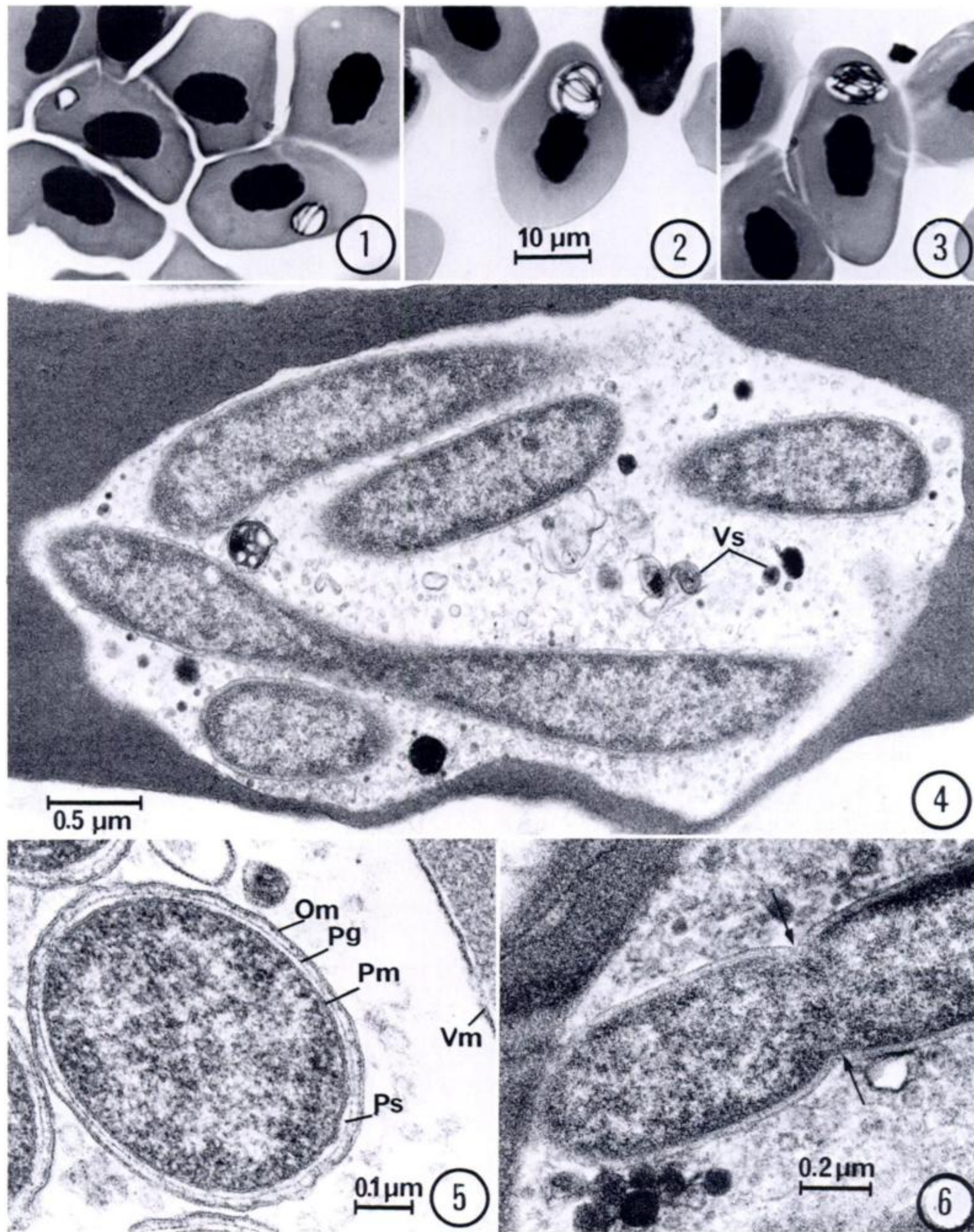


FIGURES 1–3. Photomicrographs of Giemsa stained *Aegyptianella bacterifera*. 1. Immature inclusions of *A. bacterifera* within the cytoplasm of the host erythrocytes. Note the small number of organisms within distinct, translucent vacuole. 2. Larger immature inclusion containing long, folded organisms. 3. Mature inclusion containing about a dozen organisms.

FIGURES 4–6. Electron micrographs of thin-sectioned inclusions containing *A. bacterifera*. 4. The inclusions consisted of a membrane bound vacuole containing elongate rickettsiae and numerous vesicles of varying electron density. Vs, vesicles. 5. Transversely sectioned organism demonstrating a typical gram-negative cell envelope. Om, outer membrane; Pg, peptidoglycan layer; Ps, periplasmic space; Pm, plasma membrane; Vm, vacuolar membrane. 6. Evidence for binary fission within the vacuoles. Arrows, location of division furrow.

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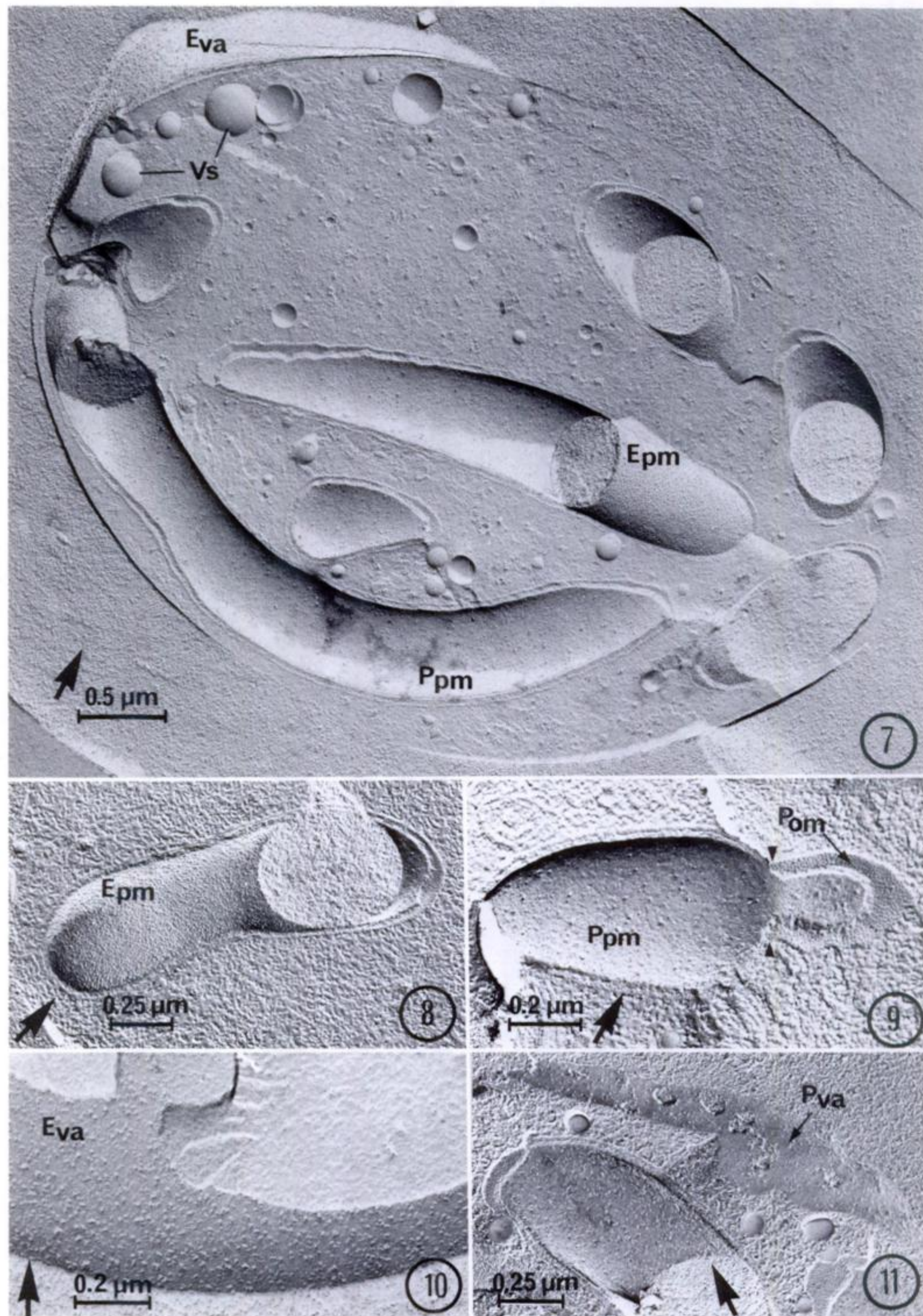


FIGURES 7–11. Electron micrographs of freeze-fracture replicas of *Aegyptianella bacterifera* within erythrocytes. Direction of platinum shadowing in each figure indicated by an arrow. 7. A mature inclusion. Numerous membranous vesicles (Vs) are visible in addition to the elongate rickettsiae. $E_{vm}$, E-face of the vacuolar membrane; $E_{pm}$, E-face of the plasma membrane of *A. bacterifera*; $P_{pm}$, P-face of the plasma

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membrane was covered entirely by closely packed IMP's of a uniform size (Figs. 7, 9). These IMP's corresponded in size and arrangement to pits on the complementary (P-face) of the outer membrane.

The vacuole surrounding the rickettsiae consisted of a single membrane. The P-face of this membrane was endowed with randomly arranged IMP's which were less densely packed than those of the E-face of the cytoplasmic membrane of the organisms (Fig. 10, cf. Fig. 8). The E-face of the vacuolar membrane was relatively free of IMP's (Fig. 11).

## DISCUSSION

*Aegyptianella bacterifera* has a gram-negative cell envelope as is typical for rickettsiae (Ristic and Kreier, 1974). The inner, plasma membrane is closely associated with dense material which is probably comprised of ribosomes. A narrow band of electron dense material between the plasma membrane and outer membrane likely represents peptidoglycan as was demonstrated histochemically for a species of the genus *Rickettsiella* (Louis et al., 1977). It appears that the membrane bound vacuole is formed during or soon after entry of an organism into its host erythrocyte. The rickettsiae increase in length and undergo binary fission until about a dozen are found in mature inclusions. Presumably, they are released through lysis of the host erythrocyte.

The fracture planes exhibited by *A. bacterifera* were typical of gram-negative organisms. The cells usually fractured between the lipid bilayers of the plasma membrane (see Fig. 7); however, fractures also occurred between the bilayers of the outer membrane. The distribution and number of IMP's within the plasma and outer membranes were similar to that exhibited by *Escherichia coli* B (Nanninga, 1970; Van Gool and Nanninga, 1971) and a *Rickettsiella* sp. (Louis et al., 1977) after freeze-fracturing. It is interesting that the *Rickettsiella* sp. has similar IMP distribution in its membranes even though it undergoes chlamydia-like development in its host cells (Louis et al., 1977).

*Aegyptianella bacterifera* and *A. ranarum* share many typical rickettsial features including their ultrastructure, mode of division and enclosure within a membrane bound vacuole in the erythrocyte. They differ strikingly, however, in that *A. bacterifera* is consistently about four times the size of *A. ranarum* and has about one tenth the number of organisms per inclusion. The occurrence of these similar, but significantly different, rickettsiae in two related host species is intriguing. Perhaps the ancestor of these rickettsiae infected the ancestor of their frog hosts and has cospeciated into two distinct *Aegyptianella* spp. along with their hosts as suggested by Barta et al. (1989).

The genus *Aegyptianella* was erected when *A. pullorum* was described from the blood of domestic birds (Carpano, 1929). Subsequently, a number of other species were described from the blood of tortoises, snakes and a variety of wild birds (Ristic and Kreier, 1974; Castle and Christensen, 1985). This report confirms that *Aegyptianella bacterifera* morphologically resembles *A. ranarum* in ranids from North America (see Desser, 1987), *A. pullorum* of birds (see Bird and Garnham, 1969; Castle and Christensen, 1985) and *Anaplasma marginale* of ruminants (see Ristic and Kreier, 1974). The morphological similar-

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membrane. 8. Fracture through the plasma membrane of a single rickettsia. Note the numerous IMP's on the E-face ($E_{pm}$). 9. Fracture through the region of a division furrow. The densely packed IMP's of the P-face of the outer membrane ($P_{om}$) contrast with the comparatively sparse IMP's of the P-face of the plasma membrane ($P_{pm}$). Arrows, location of the division furrow. 10. Fracture through the vacuolar membrane demonstrating the sparse IMP's of the E-face of the vacuolar membrane ($E_{vm}$). 11. The corresponding P-face of the vacuolar membrane ($P_{vm}$) had only a few IMP's.

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ities exhibited by these organisms support their common inclusion in the family Anaplasmataceae. However, the generic definition of the genus *Aegyptianella* must be broadened to include organisms as long as approximately 5 $\mu$m before binary fission. Although organisms this large have not been reported previously within the family Anaplasmataceae, there are examples within the genus *Rickettsia* (family Rickettsiaceae) that do attain this size.

**Acknowledgments**

Research funding was provided to S. S. Desser through a Natural Sciences and Engineering Research Council of Canada (NSERCC) operating grant #6965. Financial assistance was provided to J. R. Barta through an NSERCC Post-Graduate Scholarship. Our thanks go to John Youson of the Department of Zoology, Scarborough Campus, University of Toronto for providing the freeze-fracture facilities and Henry Hong for his help with the freeze-fracture techniques. The assistance of the Electron Microscopy Unit of the Department of Zoology, University of Toronto is also acknowledged.

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*Received for publication 27 June 1988.*

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