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Source: Journal of Wildlife Diseases, 30(2) : 169-175

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-30.2.169

Journal of Wildlife Diseases, 30(2), 1994, pp. 169–175

MORPHOLOGY, ULTRASTRUCTURE AND TAXONOMIC STATUS OF *TODDIA* SP. IN NORTHERN WATER SNAKES (*NERODIA SIPEDON SIPEDON*) FROM ONTARIO, CANADA

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ABSTRACT: Inclusions characteristic of an infection caused by *Toddia* sp. were found in the erythrocytes and erythroblasts of 15 of 26 northern water snakes (*Nerodia sipedon sipedon*) collected near Kingston, Ontario, Canada. Erythrocytes contained translucent inclusions, small acidophilic bodies, and square-shaped crystalloid structures. Erythrocytes infected with *Toddia* sp. were more rounded than uninfected erythrocytes and had pycnotic nuclei. We observed icosahedral virus particles measuring 195 to 210 nm formed from a membrane-bounded viral assembly site in the cytoplasm of the host erythrocyte. As a result of the viral identity of this parasite, we recommend that the etiologic agent of *Toddia* sp. infections from this and other species of North American snakes be renamed Snake Erythrocytic Virus.

Key words: *Toddia* sp., northern water snake, *Nerodia sipedon sipedon*, ultrastructure, icosahedral cytoplasmic deoxyribovirus.

INTRODUCTION

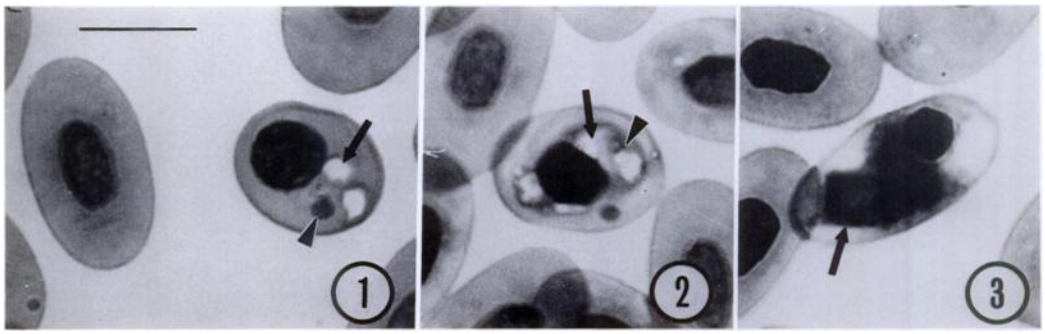
Dutton et al. (1907) first described round, red-staining inclusions associated with crystalloid bodies in the erythrocytes of African frogs. França (1911) subsequently created the genus *Toddia* to accommodate similar intraerythrocytic, acidophilic inclusions from the blood of the toad *Bufo regularis*. *Toddia* sp. was regarded as a protozoon until Marquardt and Yaeger (1967) speculated that the inclusions were the result of an infection caused by a deoxyribovirus. However, based on cytochemical tests, Arcay-de-Peraza and Diaz-Miláde-la-Roca (1971) argued that *Toddia* sp. should be classified as a protozoon. Based on ultrastructural studies of erythrocytes infected with this parasite, De Sousa and Weigl (1976) observed virus particles that resembled icosahedral cytoplasmic deoxyriboviruses. Although *Toddia* sp. now is known to be a virus (Booker and Yongue, 1982; Telford, 1984), a name consistent with viral nomenclature has not yet been given to this parasite.

Since its initial description, intraerythrocytic infections resembling *Toddia* sp. have been reported from fish, amphibians, lizards and snakes in the United States, Central and South America, central Africa and southeastern Asia (Johnston, 1975; Booker and Yongue, 1982). Our objective is to report the presence of *Toddia* sp. in the northern water snake (*Nerodia sipedon sipedon*) from Ontario, Canada and describe its morphological and ultrastructural features and cytopathological effects on the host erythrocyte. In addition, we present a review of the current taxonomic status of this virus based on our description and those of previous studies of this parasite.

MATERIALS AND METHODS

Twenty-six adult northern water snakes were collected from marshes in the vicinity of Queen's University Biological Station (44°34′N, 76°15′W) near Kingston, Ontario, Canada, during May 1992. Thin films were made from blood collected from the caudal vein, air-dried, and treated with Diff-Quik® stain (Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada). Photomicrographs were taken with a Zeiss Universal 1 photomicroscope (Carl Zeiss Canada Limited, Don Mills, Ontario, Canada) using Kodak T-MAX 100 film (Eastman Kodak Company, Rochester, New York, USA).

For transmission electron microscopy, blood from a snake heavily infected with *Toddia* sp. was drawn into a heparinized hematocrit capillary tube and centrifuged at 3,000 × *g* for 1 min. Erythrocytes were fixed in 2.5% (v/v) glutaraldehyde (Pelco International, Redding, California, USA) in 0.09 M Sörensen's phosphate buffer (pH 7.2) (Sigma Chemical Company, St. Louis, Missouri, USA) for 1 hr and post-fixed in



FIGURES 1–3. Photomicrographs of Diff-Quik®-stained erythrocytes showing different aspects of a *Toddia* sp. infection from the northern water snake, Ontario, Canada, 1992. Bar = 10 μm. 1. Translucent (arrow) and dark-staining (arrowhead) inclusions in an erythrocyte containing *Toddia* sp. The infected cell is characterized by its spheroidal shape and the presence of an eccentric, pycnotic nucleus. The adjacent uninfected cell has a central nucleus and homogeneous cytoplasm. 2. A more advanced stage of a *Toddia* sp. infection, characterized by larger translucent inclusions (arrow) and darker-staining material (arrowhead) in the cytoplasm. Note the dark-staining body and the condensed host nucleus. 3. Infected erythrocyte with a crystalloid body (arrow), surrounding flocculent material, and cleared cytoplasm.

1.0% (w/v) osmium tetroxide (Pelco International, Redding, California) in 0.13 M Sörensen's buffer containing 0.8% (w/v) potassium ferrocyanide (J. T. Baker Chemical Company, Phillipsburg, New Jersey, USA) and 0.15 M sucrose for 1 hr in the dark. The cells were dehydrated through a graded ethanol series and infiltrated and embedded in Spurr's resin (Pelco International, Redding, California, USA). Ultrathin sections were stained using 2.0% (w/v) uranyl acetate (Polysciences, Inc., Warrington, Pennsylvania, USA) in 50% methanol, post-stained in Reynolds (1963) lead citrate, and examined with a Hitachi H7000 electron microscope (Nissei Sangyo Company Limited, Tokyo, Japan).

RESULTS

Fifteen of the 26 snakes examined were infected with *Toddia* sp., with a viremia ranging from 0.1% to 28% (2,000 cells examined).

Whereas uninfected snake erythrocytes were ellipsoidal and flattened with a homogeneous cytoplasm and a central nucleus, erythrocytes harboring cytoplasmic inclusions generally were more spheroidal and contained an eccentric, condensed nucleus (Fig. 1). Three types of inclusions were seen in the cytoplasm of infected erythrocytes. The first type appeared as irregular, translucent structures which occupied a variable amount of the host cell cytoplasm (Figs. 1, 2). A diffuse, darker-staining region often surrounded these inclusions (Fig. 2). A second type of inclusion, a smaller, acidophilic body, was often found adjacent to translucent inclusions (Figs. 1, 2). A pale blue-staining square-shaped crystalloid body was less commonly observed (Fig. 3).

An increase in the volume of the cell occupied by the various inclusions was evident in what may have been an advanced stage of viral infection (Fig. 2). In addition, erythrocytes with what may have been early stages of a *Toddia* sp. infection contained only the translucent and acidophilic inclusions. Those cells that exhibited an advanced stage of infection typically harbored crystalloid bodies associated with flocculent masses of cytoplasmic material (Fig. 3).

Based on transmission electron microscopy, icosahedral virus particles, consisting of an electron-dense nucleoid surrounded by a less electron-dense hexagonal capsid, occurred in the cytoplasm of infected erythrocytes (Fig. 4). Mature virus particles measured 195 to 210 nm and were bounded by a trilaminar membrane. Pairs or clusters of adjacent virus particles were frequently enclosed by a common trilaminar envelope (Fig. 5). Virus particles appeared to form from a region on the pe-

riphery of a membrane-bounded inclusion in the cell cytoplasm (Fig. 6). The interior of this apparent viral assembly site was filled with a material that was only slightly more granular than the host cell cytoplasm. Crystalloid bodies were observed as membrane-bounded square-shaped inclusions, the interior of which had an unstructured consistency comparable to the cytoplasm of the host erythrocyte (Fig. 4). Other features of infected cells included concentric membranes found near the periphery of the cell (Fig. 7), irregular membrane-bounded masses of amorphous material similar in consistency to the cell cytoplasm (Fig. 4), and, in rare cases, massive cytoplasmic destruction accompanied by a degenerated nucleus (Fig. 8).

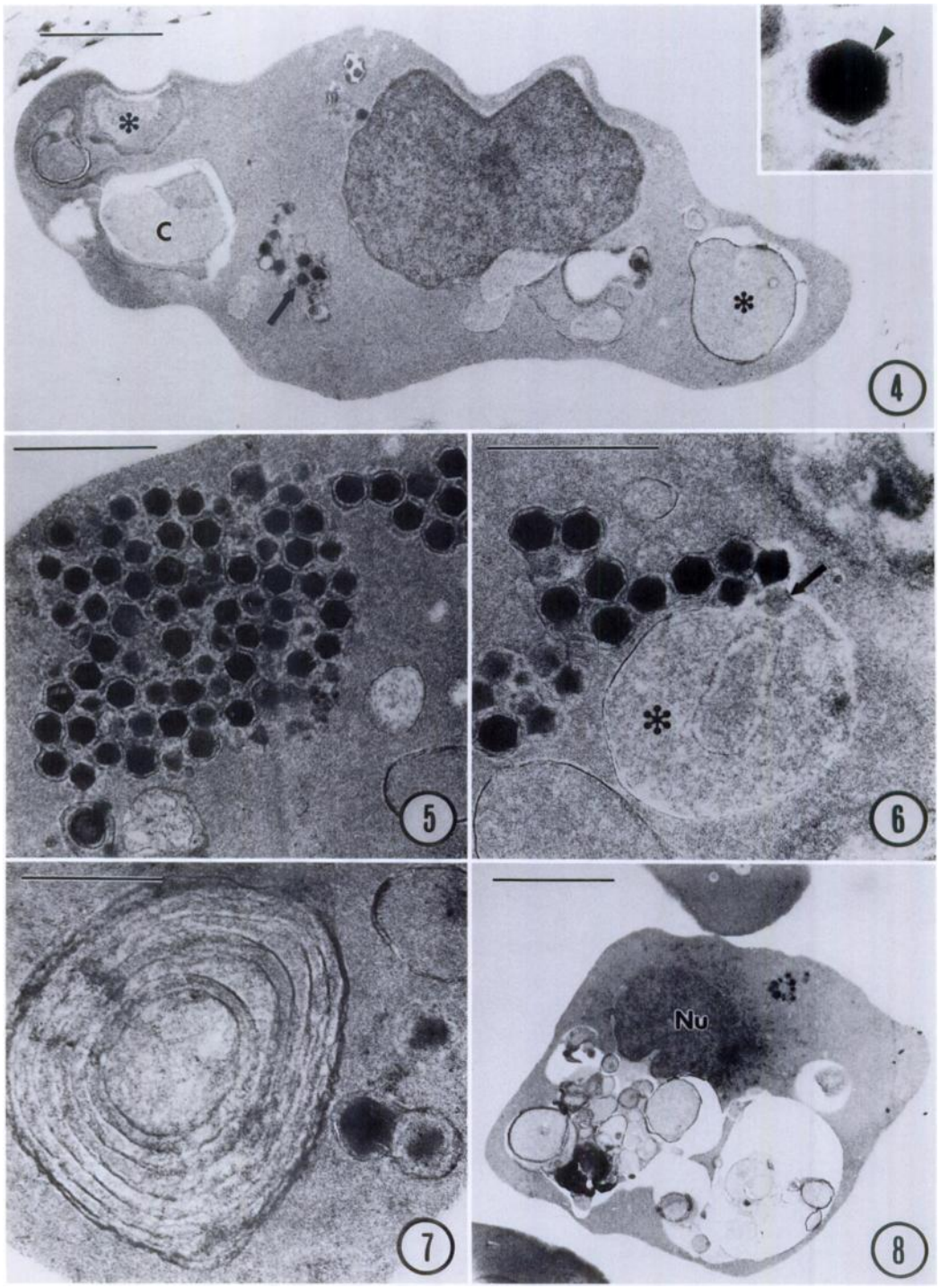
DISCUSSION

The prevalence (58%) of *Toddia* sp. in this study represents the highest recorded value to date. Only 2.5% of western cottonmouths (*Agkistrodon piscivorus leucostoma*) (Marquardt and Yaeger, 1967), 8.3% of frogs (*Leptodactylus ocellatus*) (Pereira et al., 1973), and 5.6% and 25% of northern water snakes (Booker and Yongue, 1982), from Virginia (USA) and Michigan (USA), respectively, were infected with the virus. The viremia of 28% recorded from the most heavily infected snake is similar to 29% obtained from cottonmouths (Marquardt and Yaeger, 1967) and 31% found in the viperid, *Bothrops moojeni* (De Sousa et al., 1973).

The three types of cytoplasmic inclusions were typical of *Toddia* sp. infections. The translucent inclusions seen by light microscopy were similar to the "chromatic" bodies found in the erythrocytes of *L. ocellatus* (De Sousa and Weigl, 1976), and most likely corresponded to the membrane-bounded masses of amorphous material seen in electron micrographs. The smaller acidophilic bodies associated with these inclusions were previously recorded in *Bothrops moojeni* (De Sousa et al., 1973) and in the diamondback water snake (*Nerodia rhombifer*) (Telford, 1984). These bodies, equivalent to the viral assembly site and dense clusters of adjacent virus particles observed with electron microscopy, stained red as seen by light microscopy due to the presence of DNA in both structures. This relationship was noted previously in erythrocytes infected with *Toddia* sp. (De Sousa and Weigl, 1976) and in cells with inclusions of frog erythrocytic virus (FEV) (Gruia-Gray et al., 1989).

Crystalloid structures observed by light microscopy in the erythrocytes of the northern water snake were similar to those observed by Booker and Yongue (1982) in the same species, and by Marquardt and Yaeger (1967) in the western cottonmouth. These small square-shaped bodies, found in the cytoplasm of erythrocytes, differed considerably from the large ovoid or hexagonal crystalloid inclusions found in both the cytoplasm and the nucleus of the erythrocytes of the snake *B. moojeni* (De Sousa et al., 1973) and the frog *L. ocellatus* (De Sousa and Weigl, 1976). Structural differences in the crystalloid bodies of *Toddia* sp. infecting water snakes and *L. ocellatus* were similarly apparent by electron microscopy. The crystalloid bodies described by De Sousa and Weigl (1976) from *L. ocellatus* lacked an enclosing membrane and had an internal periodicity that resembled a crystalline lattice, whereas the similar inclusion in infected snake cells was membrane-bounded and filled with an amorphous material resembling the cytoplasm of the host erythrocyte.

The dimensions of the virus particles (195 to 210 nm) found in the erythrocytes of the northern water snake were similar to those recorded by De Sousa and Weigl (1976) in the erythrocytes of *L. ocellatus* (195 to 224 nm). Virus particles in the process of forming from viral assembly sites have been previously recorded from *L. ocellatus* (De Sousa and Weigl, 1976). However, the structure of and the manner by which virus particles were formed from these sites differed from those of the northern water snake. Viral assembly sites of *Toddia* sp. found in snake erythrocytes had



FIGURES 4–8. Electron micrographs of northern water snake erythrocytes infected with *Toddia* sp., Ontario, Canada, 1992. 4. Infected cell containing virus particles (arrow), a crystalloid body (C) and several membrane-bounded inclusions (*). Bar = 2 μm. Inset of a virus particle, measuring 200 nm vertex to vertex, with an electron-dense nucleoid and a less electron-dense capsid (arrowhead) surrounded by a membrane. 5. Cytoplasm of a cell containing clusters of icosahedral virus particles bounded by individual or common

a homogenous consistency and were always membrane-bounded. Virus particles appeared to bud singly from a specific region on the periphery of this assembly site. The corresponding structures in frog erythrocytes had a granular consistency and were not bounded by a membrane. In this case, virus particles completely surrounded this amorphous structure, with partially formed particles closest to the granular material of the assembly site.

Although differing from *Toddia* sp. in size and structure, viral assembly sites, concentric membranes, crystalloid bodies, and a variety of membrane-bounded inclusions also were found in FEV (Gruia-Gray et al., 1989) and frog leukocytic virus (Desser, 1992); thus these attributes may be characteristic of icosahedral cytoplasmic deoxyriboviruses from poikilothermic vertebrates.

Toddia sp. can be differentiated by light microscopy from lizard erythrocytic virus (LEV), an icosahedral cytoplasmic virus of similar nature that infects the erythrocytes of both lizards and snakes. Erythrocytes infected with LEV contained globular albumoid vacuoles that measured up to 4 μm in diameter (Stehbens and Johnston, 1966), and lacked the crystalloid structures often observed in cells with advanced *Toddia* sp. infections (Telford, 1984). Although two or more types of intraerythrocytic viruses have been found to simultaneously infect the same host species (Speare et al., 1991), there was no evidence based on the above characteristics to indicate that LEV was present in the surveyed snakes. However, the occurrence of what appeared to be LEV in a yellowbelly water snake (*Nerodia erythrogaster flavigaster*) captured in Arkansas (USA) (Daly et al., 1980) is evidence that this virus does infect species closely related to the northern water snake.

The only features consistent in *Toddia* sp. infections in both *L. ocellatus* and northern water snakes were the presence of translucent and acidophilic inclusions and the size of the individual virus particles. With such a disparity in other features, it is probable that the inclusions and viruses historically classified as *Toddia* sp. represent two or more distinct viruses. The differences in the structure of the viral assembly sites and crystalloid bodies found in *Toddia* sp. infections from different species of snakes and frogs were especially noteworthy. The type of viral assembly site observed in *Toddia* sp. from *L. ocellatus* was similar to those reported from FEV and LEV infections (Gruia-Gray et al., 1989; Telford and Jacobson, 1993), whereas the assembly site seen in snake erythrocytes appeared unique to an ophidian host. However, whether this variability was characteristic of two types of viruses or merely a response of the snake host to infection is unclear. The variability of the crystalloid body of icosahedral viruses and hypotheses regarding its nature also have been previously documented. As postulated by De Sousa and Weigl (1976), and more recently by Gruia-Gray et al. (1989) concerning similar crystalloid bodies in erythrocytes infected with FEV, the formation of crystalloid structures might represent a host response to a viral infection, resulting in a dissimilar structure among infected erythrocytes of different groups of poikilotherms. Alternatively, the bodies may be aggregates of residual viral protein, a hypothesis supported by differential interference contrast microscopy and biochemical data (Gruia-Gray and Desser, 1992).

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membranes. Bar = 1 μm. 6. Virus particles (arrow) budding from a viral assembly site (*) in the cytoplasm of an erythrocyte. Note the similar consistency between the cell cytoplasm and the interior of the site. Bar = 1 μm. 7. Rings of concentric membranes in the cytoplasm of an infected cell. Bar = 0.5 μm. 8. An advanced stage of a *Toddia* sp. infection, indicated by the disrupted cell cytoplasm and the degenerate cell nucleus (Nu). Bar = 3 μm.

From this work and that of De Sousa and Weigl (1976), it is clear that the etiologic agent of *Toddia* sp. infections is an icosahedral virus. Hence the generic name given to this virus is inappropriate according to the International Code of Zoological Nomenclature. Telford and Jacobson (1993) recently recommended that the generic name *Pirhemocyton* not be used to describe infections caused by LEV after they provided unequivocal evidence of its viral identity. However, while LEV is predominantly a parasite of lizards and shows similar characteristics in the erythrocytes of snakes (Daly et al., 1980), the broad host and geographic range of *Toddia* sp. makes following this example more difficult. However, the inclusions of snake erythrocytes observed in this study and in those by Marquardt and Yaeger (1967), Booker and Yongue (1982) and Telford (1984), all of which were carried out in eastern North America or northeastern Mexico, were very similar with regard to size, shape, and location in the host cell; and, although a lack of ultrastructural evidence makes it difficult to ascertain with certainty, they were likely the result of an infection caused by the same virus. As a result, we recommend that this virus of snakes from North America be referred to as Snake Erythrocytic Virus (SEV). However, further ultrastructural studies and host and geographic data will be required before the taxonomic status of *Toddia* sp. reported from anurans and ophidians from other continents can be fully resolved.

ACKNOWLEDGMENTS

We thank Don Martin and Mark Siddall for their criticisms of earlier drafts of this paper. Special thanks are due Pat Weatherhead, Mark Forbes, Francis Barry and Kent Prior for their assistance in collecting and processing snakes at the Queen's University Biological Station. This research was supported by Natural Sciences and Engineering Research Council of Canada grant #6965 to SSD and an Ontario Graduate Scholarship to TGS.

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Received for publication 28 June 1993.