Ultrastructure of *Theileria*-infected blood cells of loggerhead turtles

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The authors are grateful to Drs. Stacy and Harvey et al. for their comments concerning *Caretta caretta* theileriosis. It gives us an opportunity to clarify and expand our statements as well as to offer further micrographic evidence relative to understanding this condition. This report regarding theileriosis is based on observations that were made over a period of about 12 years and includes an analysis of multiple hematological parameters derived from a large number of *C. caretta* obtained from inhabitants of the Mediterranean Sea (see Basile et al. 2012). In spring 2011, two specimens of *C. caretta*, completely covered by leeches, were brought to the Turtle Center of the Zoological Station of Naples. The leeches were recognized as *Ozobranchus margo*, well known as turtle leeches (see figure in Di Santi et al. 2012).

The diagnostic process of theileriosis began with the detection of eosinophilic leukocytosis (Fig. 1a, Di Santi et al. 2012, and here in Fig. 1). It is well acknowledged that eosinophilia is a very frequent manifestation of parasitic infection in vertebrates. The first notable observation that was made was the presence of bacillary rod-bayonet-shaped inclusions in erythrocytes (“the most useful guide to the genus *Theileria*,” Kreir 1977) followed by their recognition in leukocytes. An abundant number of techniques were utilized to identify the

parasite. The first step was to conduct weekly hemograms on the two specimens which embraced morphologic analysis of blood films for parasitized cells (Figs. 2–7, 8–9, 10–12, and 13). In addition, samples were preserved in EPON preparations to study them under TEM (see enclosed unpublished photographs of Figs. 14–15, 16–17, 18–19, 20–21, 22–25, and 26–27). During the stage of infection, osmotic fragility tests were conducted to establish whether the parasitic infection resulted in an alteration of erythrocytic osmotic fragility. When the stage of infection visually affected the leukocytes in the blood smears, buffy coat preparations of both subjects were prepared and cultured. The results of these tests confirmed the presence of an

Fig. 1 / Eosinophilia in blood smears of parasitized *C. caretta*: two eosinophilic granulocytes (arrows) in the same microscopic area. MGG stain (A. Pica)

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Figs. 2–7 2–3 Cow erythrocytes (*p*) contain rod and racquet piroplasms (arrows) (Stockham et al. 2000). 4 Single *Theileria buffeli*-like organisms in two erythrocytes in blood from a pregnant Simmental cow. Wright-Giemsa stain (see Harvey 2011, page 43). 5 *Theileria cervi* organisms in erythrocytes in blood from a white-tailed deer. Several drepanocytes (sickle erythrocytes) are present. Wright-Giemsa stain (see Harvey 2011, page 43). 6–7 Rod and racquet piroplasms (arrows) in *Caretta* erythrocytes. MGG stain (A. Pica)

Figs. 8–9 8–9 Giemsa-stained blood smears of infected cattle: extra- and intracellular schizont forms of *Theileria annulata* parasites. Intraerythrocytic piroplasm forms are also evident ($\times 1000$) (Hassan et al. 2012)

Figs. 10–12 10–12 *Caretta* lymphocytes infected by *Theileria*. MGG stain (A. Pica)

Fig. 13 13 *Theileria annulata* schizonts in lymphoblastoid cells. Giemsa stain. $\times 1000$ (Mirzaei 2007, <http://parasitol.kr/journal/view.php?number=194>)

TEM OF THEILERIA PARASITIZED RBCs: CARETTA Vs COW

Figs. 14–15 First step: *Theileria* piroplasm in *Caretta* RBCs (A. Pica)

intense proliferation of transformed cells containing parasite particles over a period of several months (as reported, Di Santi et al. 2012). A molecular study of the parasite would have been executed as a final step, if the parasite were not recovered from the infected turtles. However, the cytologic evidence of the parasite

Figs. 16–17 *Theileria* piroplasm in an infected cow's RBCs (Stockham et al. 2000)

THEILERIA MEROZOITES: CARETTA Vs HORSE & CATTLE

Second step: parasitization of *caretta* leukocytes. A.PICA

Figs. 18–19 18 An external merozoite and 19 a merozoite entering a *Caretta* leukocyte (arrows) (A. Pica)

Figs. 20–21 20 *Theileria parva* infecting a lymphocyte (Don W. Fawcett (1987)/Science Photo Library). 21 A sporozoite (S) entering a bovine lymphocyte (Shaw et al. 1991)

disappeared from the blood smears of both subjects after 4 months of veterinarian therapy as reported by Di Santi et al. (2012).

In summary, the aggregate of observations initiating with the leech infestation, including TEM observations, which were not included in the original discussion in

Figs. 22–25 22–24 Vacuolization of *Theileria* schizonts in *Caretta* leukocytes after therapy (Di Santi et al. 2012). 25 Treatment of schizont-infected bovine cells with brefeldin A leads to a rapid vacuolization (<http://www.ilri.org/InfoServ/Webpub/fulldocs/ILRADre1993v11n1/these.htm>)

Figs. 26–27 A curved form of *Theileria* in a *Caretta* leukocyte (A. Pica)

Figs. 28–29 Blood smears of a parasitized real python. Artifacts are indicated by arrows (A. Pica)

order to keep the length of the report within practical limits (but enclosed here), demonstrate *Theileria* infestation of the two *C. caretta*. It also illustrates the positive response to medical therapy. Many workers have grappled with such a diagnosis using only morphologic observations (Collins et al. 1986; Stockham et al. 2000; Lainson 2007 as well as Harvey 2011).

Regarding the possibility that the observed inclusions were artifacts, it should be kept in mind that the recognition of the parasite was made on the basis of abundant cytologic findings (at LM and TEM), their cyclic nature, the clonal expansion of infected cells in culture, and their final disappearance from the affected turtles. The capacity of this parasite to cause host cell transformation is unique among eukaryotic pathogens. In fact, as textually reported by Marsolier et al. (2015), “*Theileria* is the only Genus that transforms its mammalian host cells.” In addition, the detection of active erythrophagocytosis in circulating blood is another recognized phenomenon in response to parasitization (Jaensch and Raidal 2006; Ruggiero et al. 2014). Moreover, *Theileria*-infected cells were detected and photographed in ideal areas in the center of microscopic fields and not in thick-smear regions. As an example, artifacts observed in red blood cells in smears of a python by our research group are attached below (Figs. 28 and 29).

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