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Tetrodotoxin does not protect red-spotted newts, *Notophthalmus viridescens*, from intestinal parasites

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ABSTRACT

Tetrodotoxin (TTX) and its analogue 6-*epi*TTX had been detected in the red-spotted newt, *Notophthalmus viridescens*. Thirty specimens of a population from Pennsylvania, USA were histologically examined for the presence of intestinal parasites. More than 50% were found to be infected with nematodes (Trichocephalidae), trematodes or cestodes (Pseudophyllidae). The mean values of TTX and 6-*epi*TTX in parasitized and in non-parasitized newts were not significantly different. Using a monoclonal antibody-based immunoenzymatic technique, TTX was localized in the intestinal tissue as well as in the parasites indicating that they accumulate the toxin and suggesting that TTX and 6-*epi*TTX are not providing protection from parasites to the newts.

1. Introduction

Tetrodotoxin (TTX) and its analogues 6-*epi*TTX and 11-oxoTTX have been detected in the efts and adults of the red-spotted newt, *Notophthalmus viridescens*, from the eastern part of North-America (Yotsu-Yamashita and Mebs, 2001, 2003). Remarkably high concentrations of TTX were found reaching levels of more than 23 μg per g bodyweight. Using a monoclonal antibody-based immunohistological technique, TTX was localized in the granular glands of the newt's epidermis as well as in most of its organs, particularly in the intestinal tract (Mebs et al., 2010).

The role TTX may play in the chemical defence against predators has been extensively studied in rough-skinned newt *Taricha granulosa* from the west-coast of the United States (Brodie and Brodie, 1991; Feldman et al., 2009). A coevolutionary arms race has been suggested to occur between the newt and its major predators, i.e. garter snakes (*Thamnophis* spp.; Brodie et al., 2002, 2005; Hanifin et al., 2008; Williams et al., 2010). Whether this also applies to *N. viridescens* is an open question.

Amphibians are infested by various kinds of endoparasites like helminths (Prudhoe and Bray, 1982; Yoder and Coggins, 2007) as well as of ectoparasites such as leeches. In *N. viridescens*, Gill (1978) noted that leeches appear to be the major source of adult mortality. Newts of Virginia populations were observed to move on land to remove infestations of leeches by trying to bite and scratch them from their body. Since red-spotted newts from Virginia have been shown to contain high levels of TTX (Yotsu-Yamashita and Mebs, 2001, 2003), the toxin has obviously no protecting effect in deterring ectoparasites like leeches.

In the present paper red-spotted newts were examined for intestinal parasites. The levels of TTX and of its analogues were assayed in the newt's body and the presence of the toxin in the intestinal tissue and in the parasites is demonstrated using monoclonal antibody-based immunohistochemical methods.

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2. Materials and methods

2.1. Newt specimens

Adult specimens of *N. viridescens* were collected near the pond and in the forest of the Powdermill Research Station, Rector, Westmoreland Co., Pennsylvania, USA in September 2010. The newts were killed by freezing and after dissection the intestines were placed in buffered formalin for histological processing, the rest of the body in 70% methanol containing 0.1 vol. % acetic acid for TTX-extraction.

2.2. Toxin assay

The methanolic extracts were evaporated to dryness at 30 °C in a stream of nitrogen. Each dry residue was dissolved in 0.05 M acetic acid (1.0 ml/g newt), centrifuged and a part of the supernatant was filtered through a Cosmospin filter H (0.45 μm, Nakalai Tesque, Kyoto, Japan). An aliquot (2 μl) of the filtrate was applied to a post-column LC-fluorescent detection (LC-FLD) (Yasumoto and Michishita, 1985; Shoji et al., 2001) for the analysis of TTX and of its analogues. For standard, TTX and 6-*epi*TTX were isolated from the puffer fishes (*Fugu poecilonotus* and *Fugu pardalis*) and the newt *Cynopus ensicauda*, respectively, and quantified by ^{1}H NMR spectroscopy (Yotsu-Yamashita et al., 1999). These analogues in the sample solutions were quantified based on the area size of their peaks on the LC-FLD chromatograms.

2.3. Histological and immunohistological analysis

After fixation in buffered formalin the intestines of the newts were embedded in paraffin and cross-sections (5 μm thickness) were done. For examining the tissue for parasites, trichrome staining was first applied. For immunohistological staining each section was treated with 1% H_2O_2 and 25% goat serum and was incubated with monoclonal anti-TTX antibody (Kawatsu et al., 1997) followed by biotin-conjugated anti-mouse IgG (Sigma–Aldrich, St. Louis, Mo, USA) using the streptavidin and biotinylated horseradish complex kit (Dako Cytomation, Hamburg, Germany) and 3.3′-diaminobenzidine (DAB) as substrate. The antigen (TTX)-antibody complex was visualized as brown colour deposits. The sections were counterstained with heamatoxylin. For negative control mouse serum was used instead of the anti-TTX antibody.

3. Results

3.1. Identification of intestinal parasites in the newts

Histological analysis of cross-sections of the intestines of 30 newt specimens revealed that 16 contained parasites in their intestinal tract belonging to three orders: Nematoda (Trichocephalidae) in 9, Trematoda in 4 and Cestoda (Pseudophyllidae) in 4 specimens, respectively (Fig. 1). A precise species diagnosis for the cestodes was not possible. The intestines of 14 newts were entirely free of parasites.

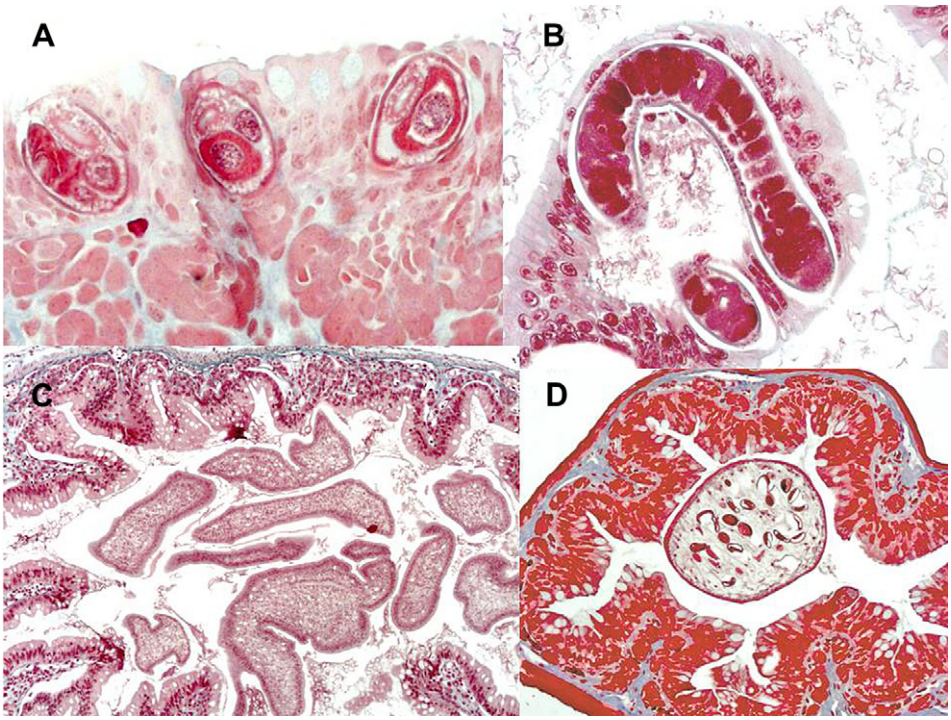

Fig. 1. Intestinal parasites of the red-spotted newt, *Notophthalmus viridescens*. (A, B) Nematodes belonging to the order Trichurida, probably of the genus *Capillaria*, which typically live inside tissues, not in the intestinal lumen; in (B) with a prominent stichosome. (C) Cestode (larval stage?) (D) Trematode (operculated eggs). Trichrome staining.