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Original Article

Morphological and molecular identification of *Corynosoma caspicum*, and its histopathological effect on the intestinal tissue of a Caspian seal (*Pusa caspica*)

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Abstract

Background: *Corynosoma* is a parasite from the Acanthocephala phylum with worldwide distribution. *Corynosoma* parasites are found in pinnipeds as their definitive host. **Aims:** This study aimed to investigate the morphological and molecular characteristics of *Corynosoma*, and its histopathological effect on the intestinal tissue of *Pusa caspica*. **Methods:** A severe *Corynosoma* infection was observed in the small intestine of a juvenile male Caspian seal (*P. caspica*). The morphological descriptions were done using light microscopy, and scanning electron microscopy (SEM). The molecular diagnosis was performed using partial sequences of internal transcribed spacer 1 (ITS1) and 5.8S ribosomal RNA (*rRNA*) gene. **Results:** According to the results, the *Corynosoma* specimens were identified as *Corynosoma caspicum.* The histopathological inspection of intestinal tissue revealed lesions in epithelial cells, mucosa, submucosa and muscle layers, destruction of intestinal glands, and infiltration of inflammatory cells. **Conclusion:** Presence of such a severe infection in one of the individual Caspian seals can suggest the possibility of morbidity among other seals in the landlocked Caspian Sea. Thus, further research on their parasite infections is required for understating the status of the Caspian seal population and conserving this endangered species.

Key words: Caspian Sea, *Corynosoma caspicum*, Histopathology, Molecular, *Pusa caspica*

Introduction

Corynosoma Lühe (1904) is a genus from *Acanthocephala phyllum* (Aznar *et al*., 2006; Schmidt, 1985) that exhibits a complex life cycle including amphipods as intermediate hosts, fish as paratenic hosts, along with marine mammals, waterfowl, and seabirds as definitive hosts (Amin *et al*., 2011). *Corynosoma* is a zoonotic parasite that can also infect humans by eating fish (Fujita *et al*., 2016).

Pinnipeds are the original definitive hosts for *Corynosoma* (Hernández-Orts *et al*., 2006). Various species of *Corynosoma* have been reported in pinnipeds such as *C. strumosum* and *C. magdaleni* from Saimaa ringed seals (*Pusa hispida*) and grey seals (*Halichoerus grypus*) (Nickol *et al*., 2002; García-Varela *et al*., 2005), and *C. australe* from South American fur seal (*Arctocephalus australis*) (Silva *et al*., 2014).

Caspian seal, *Pusa caspica* Gmelin (1788) is the only mammal of the Caspian Sea which belongs to Pinnipedia and can be the specific definitive host for *Corynosoma* (Kurochkin, 1975). Caspian seal is listed as an endangered animal by the international union for the conservation of nature (IUCN) Red List (Sharipov, 2012; Goodman and Dmitrieva, 2016). The number of Caspian seals in 1867 was estimated to be at least 1 to 1.6 million, but their population declined by 90% to about one hundred thousand seals in 2005. The status of the only Caspian Sea mammal changed from vulnerable to endangered in October 2008 (Harkonen *et al*., 2008; Harkonen *et al*., 2012).

Caspian seals have a regular immigration pattern from the north to the south part of the Caspian Sea (Perrin *et al*., 2009; Sanaee *et al*., 2020). They give birth in the frozen north in late winter and then migrate to the south deep waters to find food (Krylov, 1990; Miyazaki, 2001; Dmitrieva *et al*., 2016). The Caspian seals feed on fish, and the infected fish can transfer individuals of *Corynosoma* to the Caspian seals. Species of *Corynosoma* mature and reproduce in the gastrointestinal tract, and can cause intensive damages to the stomach or/and intestine (Amin *et al*., 2011).

Two species of *Corynosoma* including *C. caspicum* and *C. strumosum* have been reported from the Caspian Sea so far. *Corynosoma caspicum* has been isolated from *Acipenser stellatus*, *Acipenser guldenstaedti* and *Huso huso* (Golvan and Mokhayer, 1973), *Gasterosteus aculeatus* (Rahimi-Esboei *et al*., 2017), and *P. caspica* (García-Varela *et al*., 2005; Aznar *et al*., 2006), as well as *C. strumosum* from *A. gueldenstaedti* and *H. huso* (Sattari and Mokhayer, 2005), *Neogobius fluviatilis pallasi*, *N. kessleri gorlap* and *N. bathybius* (Pazooki *et*

al., 2011), and *P. caspica* (Kurochkin, 1975; Amin *et al*., 2011).

The variety of hosts and geographical distribution lead to *Corynosoma* diversity worldwide (García-Varela *et al*., 2005). Although species of *Corynosoma* in different hosts can be identified by morphological analysis, the large number of *Corynosoma* species and the subtle differences among them complicate the identification (Golvan, 1994; Monks, 2001). Markers such as ribosomal RNA (rRNA) including the internal transcribed spacers 1 and 2 (ITS1 and ITS2) and 5.8S rRNA can be used for accurate differentiation of very similar species (García-Varela *et al*., 2005; Omidzahir *et al.,* 2015; Mousavi *et al*., 2013).

In the present study, a severe infection of *Corynosoma* was observed in the small intestine of a juvenile male Caspian seal. Morphological and molecular descriptions of *Corynosoma* specimens were investigated. Also, the infection was examined by the histopathological observations.

Materials and Methods

Collection of the specimens

One juvenile male Caspian seal with 86 cm standard body length measuring from nose to tail was found dead due to choking in a sturgeon gill net, in Miankaleh peninsula, Southeast of Caspian Sea, Iran (36°54′02.36″N, 53°47′26.28″E). Upon necropsy, a large number of *Corynosoma* were observed in the small intestine and worm specimens (n=50) were collected randomly for further analysis. Voucher specimens were deposited in the Iranian National Parasitology Museum (INPM), Veterinary Faculty, University of Tehran, Tehran, Iran.

Morphological analysis

Representative specimens of *Corynosoma* were stained by acetocarmine and dehydrated with ascending ethanol series, cleared in xylene, and then mounted on slides with Canada balsam. Then, each specimen was observed through a light microscope (Olympus CX21, Japan) equipped with a digital camera (Tucsen TrueChrome Metrics, China), and morphological features were measured via IS Capture imaging software. Scanning electron microscopy (SEM) was also used for morphological description. The specimens were prepared by critical point drying, mounted on SEM stub, and coated with gold (Goldstein *et al*., 2017). Finally, the images were observed using SEM (SNE-4500M, Korea) at a voltage of 20 kV in a connected computer with digital imaging software.

Molecular analysis

The *Corynosoma* specimens (n=10) were preserved in 70% ethanol for molecular characterization. A pair of primers were designed using the present data on *Corynosoma* species in the GenBank. The primers included sense (5´-TCG AGT TCT GCA CGA ATA AT-3´) at ITS1 and antisense (5´-TGT ACA CTG AAC ATT CAC GT-3´) at 5.8 *SrRNA* gene.

Polymerase chain reaction (PCR) was performed on 100 µL samples containing 15 µL of DNA extract from specimens, 20 pg of primers, 10 µL of PCR buffer (Cina gene, Iran), 100 mM of each dNTP, 1.5 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase (Fermentas). Thermal cycling setting included: step one for 5 min at 95°C, step two for 45 s at 94°C, 45 s at 50°C, 45 s at 72°C for denaturation, annealing, and extension, respectively; and the last step was set for 10 min at 72°C in a thermocycler (MWG, Germany). Finally, the PCR products were run on 1.5% agarose gel and observed in UV illuminator (Bio-Rad, USA). The PCR products were sequenced from both sense and antisense primers.

Histopathological study

The specimens of infected small intestinal tissue were dissected and fixed in 10% buffered formalin (Merck, Germany). The specimens were prepared in the tissue processor, with serial sections of formalin-fixed, paraffin-embedded samples cut in a rotary microtome (Leitez 1512, Germany) at 5 µm, and used for standard haematoxylin and eosin (H&E) staining. Thereafter, the sections were cover slipped and examined by light microscopy (Olympus CX21, Japan) for further histopathological inspection (Bancroft and Gamble 2001).

Results

Morphological description

Descriptions were performed based on gravid females (n=12) and adult males (n=12). Males and females were similar to each other, but the females were slightly larger. The entire body of an individual *Corynosoma* is shown in Figs. 1A and 2B. Except for the length and width of the trunks in millimeters (mm), the other measurements are in micrometers (µm) with the mean value in parentheses. Proboscis 506-682 (613) long, 232-256 (243) wide in female, and 453-611 (537) long, 209-229 (218) wide in male with a cylindrical shape and a swollen part adhered to the neck (Figs. 2A and 3A). Proboscis with 16 longitudinal rows (Fig. 3B) including 9-10 hooks per row (Fig. 2A). Upper 4 hooks measured 51-61 (57) long, 11-15 (14) wide in root in female and 44-55 (49) long, 10-13 (11) wide in male. Fifth hooks 68-82 (77) long, 23-25 (24) wide in root in female, and 65-76 (69) long, 21-23 (22) wide in male were far larger than the other hooks located in the swollen part of proboscis (Figs. 2A and 3A). Posterior hooks 35-40 (38) long in female and 32-35 (33) long in male were smaller than the others farther away from fifth hooks (Figs. 2A and 3C). Proboscis receptacle 1197-1285 (1247) long, 289-329 (315) wide in female, and 998-1195 (1076) long, 258-289 (272) wide in male were at the base of proboscis. Short conical neck 285-315 (302) long, 318-343 (332) wide in female, and 255-280 (266) long, 278-316 (294) wide in male could retract into the anterior trunk.

Trunk, 3.54-4.16 (3.93) long in female and 3.05-3.46 (3.17) long in male included anterior and posterior parts. Swollen shape anterior trunk 1.23-1.36 (1.29) wide in female and 1.04-1.22 (1.13) wide in male covered by spines 31-39 (35) long in female and 28-31 (29) long in male, anteriorly (Figs. 3B and 3D) and 26-33 (29) long in female and 23-27 (25) long in male, posteriorly that continued to the upper part of posterior trunk as shown by arrow in Fig. 1B. Posterior trunk 0.456-0.538 (0.509) wide in female and 0.376-0.499 (420) wide in male with a narrow cylindrical shape ended at genital pore (Fig. 3E) that surrounded by small genital spines 20-23 (22) long in female and 15-20 (17) long in male (Fig. 3F). In

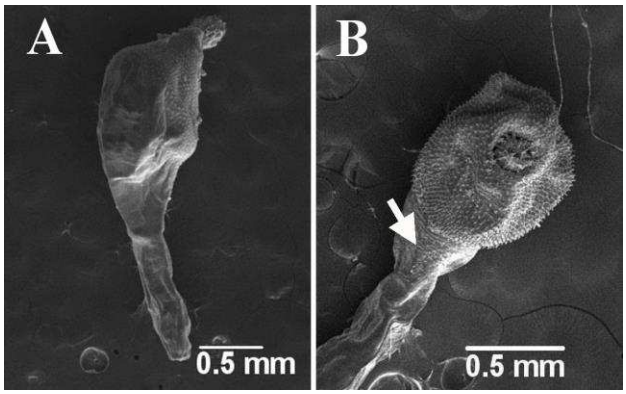

Fig. 1: Scanning electron micrographs of *Corynosoma caspicum* isolated from *Pusa caspica*. (**A**) Lateral view of entire body of *C. caspicum* (scale bar, 0.5 mm), and (**B**) Ventral view of *C. caspicum*, arrow shows the spines of anterior trunk continue to upper part of posterior trunk, (scale bar, 0.5 mm)

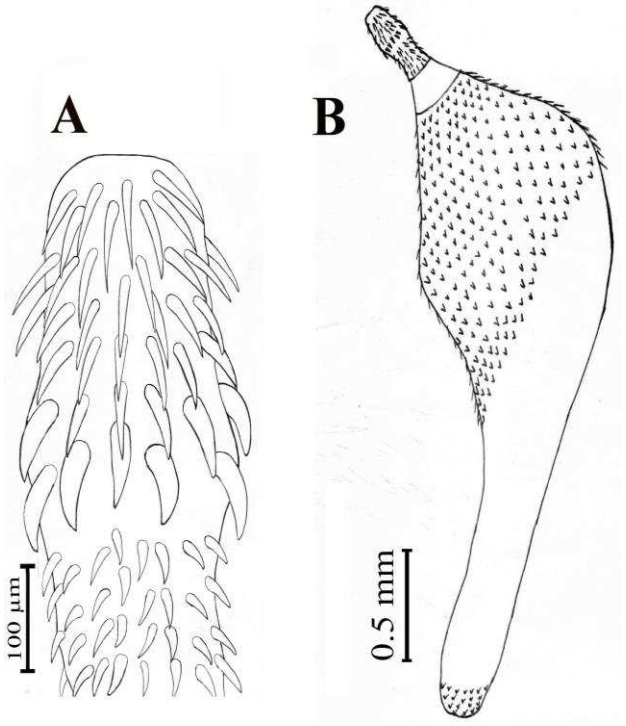

Fig. 2: Drawings of *C. caspicum* (**A**) Proboscis showing hooks per row (scale bar, 100 µm) (**B**) Entire body of *C. caspicum* showing trunk shape and distribution of spines (scale bar, 0.5 mm)

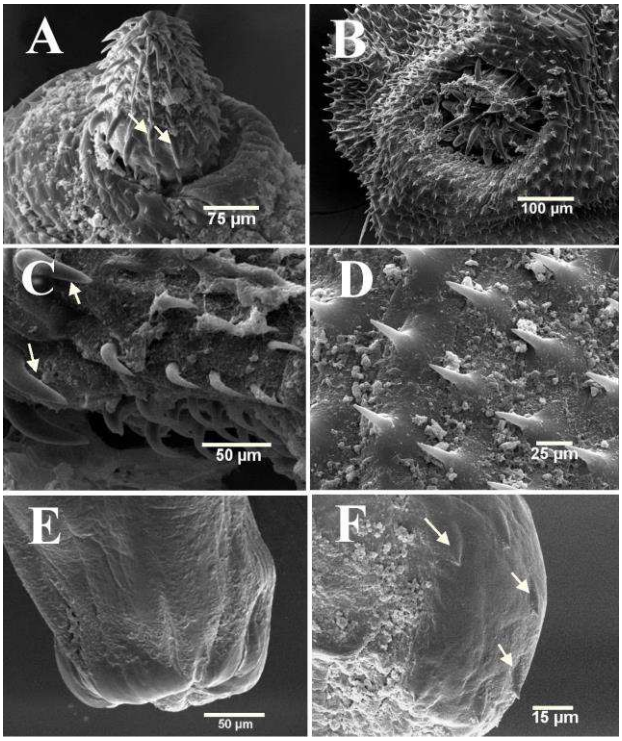

Fig. 3: Scanning electron micrographs of different parts of *Corynosoma caspicum* isolated from *Pusa caspica*. (**A**) Proboscis with a cylindrical shape and a swollen part, arrows point to fifth hooks (scale bar, 75 µm), (**B**) Apical view showing 16 longitudinal rows of hooks in proboscis and spines in anterior trunk (scale bar, 100 µm), (**C**) Arrow points to fifth hooks farther away from latter hooks (scale bar, 50 µm), (**D**) Anterior trunk spines (scale bar, 25 µm), (**E**) Posterior end of *C. caspicum* (scale bar, 50 µm), and (**F**) Posterior end, arrow points to genital spines (scale bar, 15 µm)

females, full grown eggs measured 86-115 (94) long and 22-27 (24) wide. In males, two oval testicles 196-432 (311) long, 115-285 (212) wide in the hind ending of anterior trunk, and six elongated cement glands continued after testes.

Molecular description

A nucleotide sequence with 273 base pairs (bp) was obtained from each representative *Corynosoma* specimen. The nucleotide sequences were compared with other *Corynosoma* sequences available in GenBank by abasic local alignment and search tools (BLAST). The result revealed that the nucleotide sequences were identical with 100% similarity to *C. caspicum* under accession number AF286309 (Garcia-Varela *et al*., 2005). Molecular analysis indicated that all parasite specimens in this study belonged to *C. caspicum.* The nucleotide sequence of the *Corynosoma* species in the present study was deposited in the GenBank as *C. caspicum* under accession number MF374457.

Gross and histopathological findings

Gross examination showed severe infection throughout the small intestine of the Caspian seal. In total, more than 500 *Corynosoma* were estimated in the

small intestine. The *Corynosoma* parasites were attached to the intestinal wall or free in the lumen of the intestine. Due to the parasite infection, the tissue color was changed to dark red (Fig. 4A).

Histopathological findings indicated notable lesions. The intestinal villi were damaged and significant atrophy was observed (Fig. 4B). Aggressive parasites are attached with their proboscis to the intestinal wall. As presented in Fig. 4C, the migration route of the parasite into the intestinal tissue and damage of the mucosa and submucosa layers was demonstrated. Epithelial cells and connective tissue cells displayed necrotic lesions. Intestinal glands were also disturbed (Figs. 4C and E). The large number of *Corynosoma* and subsequent intestinal tissue damage caused the infiltration of inflammatory cells (Figs. 4C-E).

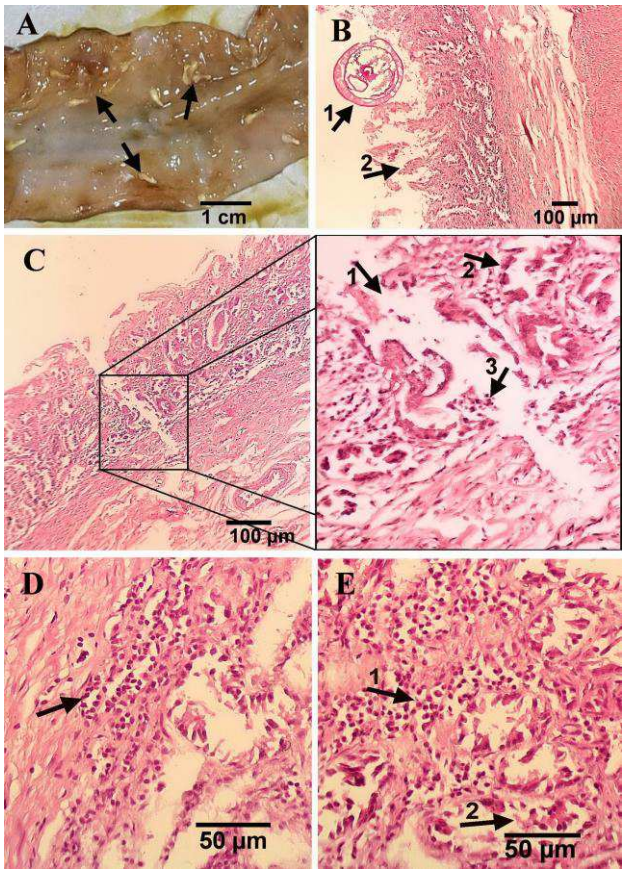

Fig. 4: Macroscopic and microscopic observations of intestine of the Caspian seal infected with *C. caspicum*. (**A**) An infected intestinal section, arrows point to parasites (scale bar, 1 cm), (**B**) 1: A parasite section in the intestinal tissue, 2: Destruction of the villi subsequent parasite invasion (scale bar, 100 µm), (**C**) Damage of mucosal and submucosal layer following the migration of the parasite into the intestinal tissue (scale bar, 100 µm); 1: The migration route of parasite, 2: Destruction of the intestinal gland, and 3: Infiltration of inflammatory cells, (**D**) Inflammatory cells infiltration in submucosa (scale bar, 50 µm), and (**E**) 1: Inflammatory cells infiltration in mucosa, and 2: Destruction of intestinal gland (scale bar, 50 µm)

Discussion

The present study was conducted to identify the *Corynosoma* species in the Caspian seal by morphological and molecular analysis. Detailed morphological features presented were consistent with the species *C. caspicum*.

The original morphometric description of *C. caspicum* isolated from sturgeon as paratenic host was reported by Golvan and Mokhayer (1973). They reported an average trunk length of 3.925 mm in males and 4.675 mm in females. The females did not contain fully grown ripe egg and 4 elongated club cement glands were observed in males. The proboscis consisted of 16 or more, but rarely 18, longitudinal rows with 10 hooks in each row, and the 5th hooks were larger than the others. Furthermore, the spines of the anterior trunk continued to the upper part of the posterior trunk (Golvan and Mokhayer, 1973). In the present study, the morphological features of specimens closely resembled those of the *C. caspicum* described by Golvan and Mokhayer (1973), with the difference being that the *Corynosoma* parasites were isolated from the Caspian seal as the definitive host with fully grown eggs in gravid females and six cement glands in males.

Two species of *C. caspicum* and *C. strumosum* have been reported from the Caspian Sea. *Corynosoma caspicum* has been observed only in the Caspian Sea; however, *C. strumosum* has been isolated from *H. grypus* and *P. hispida* in the cold water of Baltic Sea (Nickol *et al*., 2002; García-Varela *et al*., 2005).

In comparison, *C. caspicum* and *C. strumosum* are similar in many morphological features such as the shape of proboscis and neck, bare area of anterior trunk, distribution of trunk spines, arrangement of testes, and number of cement glands (Aznar *et al*., 2006). Very minor differences exist between these two species. For example, trunk length of *C. strumosum* is larger than *C. caspicum* (5-7 to 9 mm vs. 3-4.7 mm). An exception was reported by Amin *et al*. (2011) where the trunk length of *C. strumosum* was measured 2.75-4.75 mm. The number of longitudinal rows of hooks in proboscis varies from 16 to 18 in both species; however, 16 rows in *C. caspicum* and 18 rows in *C. strumosum* have mostly been described.

Because of the slight morphological differences, a combination of morphological and molecular analysis was performed to confirm *Corynosoma* species in the present study. Prior to this study, the molecular description of *C. caspicum* isolated from Caspian seal had been presented by García-Varela *et al*. (2005), and the present study reported the second molecular description of this species. The nucleotide sequences of *Corynosoma* in the present study showed 100% similarity to the *C. caspicum* from *P. caspica* (AF286309) (Garcia-Varela *et al*., 2005), while they revealed 95.24% similarity to *C. strumosum* (AF286313) reported by Garcia-Varela *et al*. (2005) from *P. hispida.* Therefore, the two species can be separated using molecular description.

Further detailed studies on *Corynosoma* species isolated from different hosts are required to clarify the actual distribution of the different species of this parasite.

In this regard, molecular description of *Corynosoma* species along with morphological identification is highly recommended (Hernández-Orts *et al*., 2017).

This study also presents the first report of the histopathological effect of *C. caspicum* in the Caspian seal. Previously, the only available report was the histopathological effect of *C. strumosum* in the Caspian seal studied by Amin *et al*. (2011).

In this study, significant lesions were observed in the small intestinal tissue. The mucosa, submucosa, and muscle layers were damaged considerably following invasion of the large number of *C. caspicum*. *Corynosoma* parasites can attach to the gastrointestinal lumen, then proboscis retract and penetrates the gut wall of the host. Contraction of the dorsal neck retractor muscle of the parasite can pull the substratum of host tissue in the deep attachment (Anzar *et al*., 2018). The lesions caused by attachment points and movement of the parasites in the intestinal tissue layers can lead to secondary infections (Silva *et al*., 2014). Similar to our observations, the histopathology of the intestine in the Caspian seal infected by *C. strumosum* also revealed remarkable lesions in attachment site of the parasites in the mucosa and submucosa layers, damaged intestinal villi, hemorrhage, and epithelial cell necrosis (Amin *et al*., 2011). Also, infection of *C. australe* in the intestine of *A. australis* caused necrosis of mucosal layer, edema in submucosa, yet no severe inflammatory response. Further, several attachment sites in the intestinal wall made secondary infections and imperfect absorption of food material (Silva *et al*., 2014). Moreover, parasite number, encapsulation, and scar tissue formation due to replacement of collagenous fibers instead of epithelial cells in the intestinal lumen can cause obstruction, adversely affecting the digestive system and feeding of the seal (Amin *et al*., 2011).

It can be concluded that presence of such a severe infection due to a large number of worms in one of the individual Caspian seals may suggest the possibility of morbidity among other Caspian seals in the landlocked Caspian Sea. Thus, further research is necessary for understating the status of the Caspian seal population and conserving this endangered species, which should be taken more seriously.

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