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Canine echinococcosis: the predominance of immature eggs in adult tapeworms of *Echinococcus granulosus* in stray dogs from Tunisia

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Abstract

Canine echinococcosis is caused by the adult tapeworm of *Echinococcus granulosus*. As intermediate hosts, humans and livestock become infected following ingestion of eggs that are passed in the faeces of dogs. Mature eggs develop into hydatid cysts in different organs, leading to hydatid disease, which is a serious public health problem. In the present study, we investigated the proportion of mature eggs of *E. granulosus* in 140 dogs from three regions of Tunisia. The results showed the predominance of immature *E. granulosus* eggs in infected dogs and the occurrence of a small proportion of oncospheres. The ability of immature eggs to infect humans and livestock is discussed.

Introduction

Echinococcosis is a zoonotic disease of great importance. The infection is caused by the presence of *Echinococcus granulosus* tapeworms in the small intestine of dogs. This parasite is distributed in large parts of South America, East Africa, Australia, Central Europe, Central Asia and the Mediterranean littoral, including North Africa (Eckert & Deplazes, 2004; World Health Organization, 2013). In Tunisia, *E. granulosus* is geographically widespread (Aoun & Bouratbine, 2007; Lahmar *et al.*, 2013) and is the most significant zoonosis in the country, representing an important social and economic problem (Lahmar *et al.*, 2001; Majorowski *et al.*, 2005). The annual surgical incidence has been estimated at 12.6 per 100,000 habitants between 2001 and 2005 (Chahed *et al.*, 2010). The *E. granulosus* life cycle is maintained by infected dogs housing adult tapeworms. Mature *E. granulosus* tapeworms release numerous eggs that are deposited in dog faeces. These eggs are very resistant to external environmental conditions and can survive for months on pasture (Gemmell *et al.*, 1987; Thevenet *et al.*, 2005). Humans can accidentally become intermediate hosts by ingesting the eggs. The ingested eggs release an embryo (oncosphere) that migrates through the circulatory system to the various tissues, to develop into a hydatid cyst over many months. Human echinococcosis is usually asymptomatic until the cysts become large enough to damage tissues and organs. Cysts are often removed surgically but the success of their ablation depends on the location and size of the cysts (Eckert & Deplazes, 2004). Drug treatment is used in some cases of hydatid cysts before and/or after surgery, but there is no vaccine against *E. granulosus* for humans. However, vaccination is a possible method of controlling echinococcosis in intermediate and definitive hosts. In animal intermediate hosts, a vaccine known as EG95 was developed and characterized by Gauci *et al.* (2005). Up to 90 and 99% protection has been obtained in bovines after two and three vaccinations, respectively (Heath *et al.*, 2012). The protection was maintained for about 1 year after each immunization given 12 months apart. In the definitive host, significant suppression of egg development occurred after vaccination with soluble native proteins isolated from protoscoleces *of E. granulosus* (Zhang *et al.*, 2006) or with recombinant proteins from adult worms (Petavy *et al.*, 2008). In the same context of contol programmes, coproantigen tests were

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developed to detect *E. granulosus* infection in dogs (Lahmar *et al*., 2007). However, these tests could not confirm the presence of mature eggs, and their potential infectivity in dogs remains uncertain. Previous studies were mainly performed on the viability and infectivity of *E. granulosus* eggs under extreme conditions and over a long period (Thevenet *et al*., 2005). However, the number of mature (infective) eggs ingested by the intermediate host plays a key role in maintaining the life cycle of *E. granulosus* (Eckert *et al*., 2001). Moreover, the proportion of these eggs in adult worms from infected dogs is relevant to epidemiological investigations and control strategies (Craig *et al*., 2007). Therefore, the aim of the present study was to determine the proportion of mature eggs in *E. granulosus* from stray dogs living in three endemic regions of Tunisia.

Materials and methods

Collection and examination of dogs

Up to 140 stray dogs were collected from the regions of Kasserine, Feriana and Sbeitla in Tunisia. Dogs were anaesthetized by intrapulmonary injection of thiopental (Biocheme GMBH, Vienna, Austria) (10 mg/kg) and sacrificed by intravenous injection of T61 (Hoechst GmbH, Munich, Germany) (1 ml/kg). Dogs were analysed by region. Necropsy was performed under high safety conditions. Briefly, all the steps were performed in special rooms and the personnel involved in handling dogs wore protective clothing (cap, face mask, safety glasses, plastic apron, rubber gloves and boots). All laboratory material was decontaminated by deep-freezing at −80°C for 96 h. *Echinococcus granulosus* tapeworms were extracted as described by Craig *et al*. (1986). Briefly, each dog intestine was opened and soaked in normal saline (0.9% NaCl w/v) at 30°C and stirred intermittently until all worms were released. Then, worms were washed 8–10 times in saline, with repeated decantations of excess liquid, and resuspended in 50 ml of normal saline. A 0.5-ml aliquot was diluted at 1/10, and 0.5 ml was examined microscopically, with the number of adult worms counted at the same time.

Two other tapeworm species, *Taenia hydatigena* and *Taenia ovis*, were also identified, but adult worms of *E. granulosus* were separated according to their morphological characteristics and eggs were extracted using the enzymatic method, as described by Craig *et al*. (1986). Briefly, *E. granulosus* worms were incubated for 2 h at 37°C with continuous stirring, in a solution of 1% pepsin and 1% HCl in saline (pH 2) (artificial gastric fluid). Pepsinized material was centrifuged at 1000 × *g* for 5 min; the pellet was resuspended in 1 ml saline, layered on to 1 ml neat Percoll (Amersham Pharmacia, Uppsala, Sweden) and left for 5 min to allow dense debris and grit to sediment. The upper layer of eggs and pepsinized tissue was then transferred into 20 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2, then washed extensively, and finally resuspended in PBS containing 20 mM iodoacetamide, 2 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF), 2 µg/ml pepstatin A and 10 µl/ml aprotinin (all enzyme inhibitors were from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). A 0.1-ml aliquot of egg suspension was diluted at 1/500, and 0.1 ml was counted with a Neubauer's chamber. The numbers of mature and immature eggs were estimated at the same step and their proportions were calculated using microscopic and enzymatic methods. Mature eggs were distinguished under the microscope by their morphology. The mature eggs are thick-shelled and contain the oncosphere.

Extraction of mature eggs of E. granulosus

Eggs obtained as described above were incubated in 50-ml screw-cap tubes at 37°C for 45 min in a sterile solution of 1% pepsin (Sigma-Aldrich) and 1% HCl in 0.85% NaCl. After centrifugation at 500 × *g* for 5 min, the pepsin solution was decanted. Eggs were then washed three times with PBS and incubated in a sterile solution of 1% pancreatin (Sigma-Aldrich), 1% $NaHCO_3$, and 5% sterile sheep bile (artificial intestinal fluid). Oncospheres were checked every 2 min with a microscope until all of them were released from embryonic membranes. Oncospheres were pelleted by centrifugation at 1000 × *g* for 5 min. The supernatant was discarded, and oncospheres were washed three times with PBS. Oncospheres were further purified by density-gradient separation in Percoll and washed three times with PBS. The supernatant was discarded and pelleted oncospheres were resuspended in 10 ml of PBS. A 0.1-ml aliquot was diluted at 1/500 and 0.1 ml was counted in a Neubauer's chamber.

Results and discussion

Adult worms of *E. granulosus* were identified in 23.6% (33) of 140 dogs examined from Kasserine, Feriana and Sbeitla in Tunisia. This prevalence correlated well with values reported in other regions of the country (Lahmar *et al*., 2001). Up to 28.9% of 38 dogs from Sbeitla were infected with adult *E. granulosus*, compared with 25.5% of 51 dogs from Kasserine and 17.6% of 51 dogs from Feriana. Out of the 33 positive dogs examined from the three regions, up to 42.4% were infected with fewer than 100 worms and 27.3% were heavily infected with more than 10,000 tapeworms. Variations in worm burdens in dogs have also been reported by Jenkins *et al*. (2000) and Lahmar *et al*. (2001). In total, we have detected 403,000 *E. granulosus* tapeworms, spread over the three regions: 163,000 in Kasserine, 118,000 in Feriana and 122,000 in Sbeitla. Worm analyses have shown that only 20% (80,600) of *E. granulosus* worms contain eggs: 32,600 in Kasserine, 23,600 in Feriana and 24,400 in Sbeitla. Previous studies by Gemmell *et al*. (1986) and Constantine *et al*. (1998) have shown that *E. granulosus* develops acquired and induced immunity in dogs. From a total of 14 million recovered eggs, up to 5.2 million were estimated from Kasserine compared to 4.8 million from Sbeitla and 4.0 million from Feriana, all with a low percentage of mature eggs. An estimated 2 million eggs (14%) contained oncospheres, with 700,000 from each of Kasserine and Sbeitla and 600,000 from Feriana. However, the infectivity of immature eggs is crucial for enhancing transmission of *E. granulosus* in intermediate hosts. Previous work has shown that eggs can mature

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under natural conditions and hence are infective for the intermediate host (Thevenet *et al.*, 2005). This process of maturation could play an important role in the life cycle of *E. granulosus* and its dispersion. It is worth mentioning that we have not analysed canine faeces for the presence of tapeworms or eggs of *E. granulosus*. Mature eggs can be detected in terminal proglottids. Hence, dogs diagnosed as negative in the present study could pose a potential risk for the intermediate host.

The present results have shown that eggs obtained from adult *E. granulosus* worms extracted from the intestines of dogs are mostly immature. However, maturation of these immature eggs in the environment can generate infective eggs for the intermediate hosts.

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Conflict of interest

None.

Ethical procedures

This study was approved by the ethics committee at the National School of Veterinary Medicine, Sidi Thabet, Tunisia. All the procedures cited were performed under strict safety conditions.

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