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Review

Helminth therapy: Advances in the use of parasitic worms against Inflammatory Bowel Diseases and its challenges

M. MARUSZEWSKA-CHERUIYOT*, K. DONSKOW-ŁYSONIEWSKA, M. DOLIGALSKA

Department of Parasitology, Faculty of Biology University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland,
E-mail: *mmaruszewska@biol.uw.edu.pl*

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Summary

Development of modern medicine and better living conditions in the 20th century helped in reducing a number of cases of infectious diseases. During the same time, expansion of autoimmunological disorders was noticed. Among other are Inflammatory Bowel Diseases (IBD) including ulcerative colitis and Crohn's disease which are chronic and relapsing inflammation of the gastrointestinal tract. Absence of effective treatment in standard therapies effects the search for alternative opportunities. As per hygienic hypothesis increasing number of cases of autoimmune diseases is as a result of reduced exposure to pathogens, especially parasites. Thus, one of the promising remedial acts against IBD and other allergic and autoimmune disorders is "helminth therapy". Cure with helminths seems to be the most effective therapy of IBD currently proposed. Helminth therapy focuses on advantageous results that have been obtained from the clinical trials, but its mechanisms are still unclear. Explanation of this phenomenon would help to develop new drugs against IBD based on helminth immunomodulatory molecules.
Keywords: helminth therapy; *Heligmosomoides polygyrus*; Inflammatory Bowel Diseases; ulcerative colitis

Introduction

Helminths have co-evolved with their hosts over millions of years to arrive at a form of mutualism where both the host and the parasite derive some benefit from their relationship. The immunosuppression and immunoregulation induced by helminths is obviously beneficial for the parasite: it prevents the parasite being killed or expelled and improves its fitness; it also inhibits inflammatory reactions and otherwise innocuous antigens, thus benefitting the host by preventing local and peripheral pathologies generated against it (Barthlott *et al*., 2003). The absence of effective forms of treatment and the unsatisfactory causal effects of conventional therapies for autoimmune diseases has aroused interest in new forms of treatment (Chandrashekara, 2012). The key aspects of research into helminth therapy (HT) and helminth-derived product therapy (HDPT) concern the use of live helminths as treatment, as well as the characterization of the key molecules responsible for immunomodulation. These could be used as drugs to control inflammation and autoimmune diseases. HT currently seems to be the most effective therapy for autoimmune disorders (Wilson & Maizels, 2004); however, live nematode therapy remains undoubtedly controversial, especially as the mechanism of disease prevention and inhibition is unknown (Erb, 2009). In addition, the therapeutic effects of helminths are undoubtedly complex, and for this reason, the use of individual immune-active components isolated from nematode products as potential drugs is not as meaningful as previously believed.

* – corresponding author

Inflammatory Bowel Disease (IBD) Problems

Inflammatory Bowel Disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, idiopathic, and relapsing inflammation of the gastrointestinal tract. This disorder is most common in young adults, but can also develop in childhood and old age. The worldwide incidence rate of CD varies between 0.1 – 16/100,000 persons worldwide, while UC is more common and varies greatly between 0.5 – 24.5/100,000 persons, with the prevalence rate of IBD reaching up to 400/100,000 persons. However, the incidence and prevalence of the disorder are probably higher because despite the existing Montreal classification of IBD (Satsangi *et al*., 2006), precise diagnosis is limited by the lack of gold standard criteria for identification, resulting in inconsistent case ascertainment and disease misclassification (Lakatos, 2006; Molodecky *et al*., 2012).

The inflammation in UC is characterized by superficial ulcerations, granularity and a distorted vascular pattern. Histological features include an expansion of the lamina propria with inflammatory cells and crypt abscesses. There are usually no fistulae or granulomas: the typical histopathologic features of Crohn's disease. As a consequence, the symptoms of UC are a progressive loosening of bloody stools, rectal bleeding, diarrhea, tenesmus with cramping abdominal pain and a severe urgency to have a bowel movement up to 20 times a day (Brandtzaeg *et al*., 1997).

There is no effective treatment for colitis and therapy is based on encouraging long-term remission with anti-inflammatory medications. The four major classes of medication used today to treat UC are aminosalicylates, steroids, immune modifiers (azathioprine, 6-MP, and methotrexate) and antibiotics administered orally or rectally; however, all have restrictions, including side effects, refractoriness or unresponsiveness. In one-quarter to one-third of patients with UC, medical therapy is not completely successful or complications arise. Complications of UC can include bleeding from deep ulcerations and rupture of the bowel (Leitner & Vogelsang, 2016). Patients are at increased risk of colonic epithelial dysplasia and carcinoma, with an age-specific risk that is at least three times greater than that in the general population. As the risk of developing cancer increases in patients with long-term UC (7 to 10 years) with a rate of approximately 0.5 – 1 % per year, endoscopic surveillance examinations are performed annually and surgery offered for patients with ileal pouch-anal anastomosis. Over the long term, up to 25 % of those with UC will require surgery (Bernstein, 2001).

Although knowledge of UC dates back to the 19th Century, the pathogenic cause remains unknown. Its pathogenesis is believed to be associated with a deregulated proinflammatory response to commensal gut bacteria; it is restricted to the epithelial mucosa of the colon in an even and continuous distribution not related to any intestinal infection. Recent genetic studies have identified about 163 genes which are crucial in the development of IBD; most of them are common between Crohn's disease and ulcerative colitis (Cleynen *et al*., 2016). In addition, several environmental risk factors are known to be associated with IBD disease cases including diet, intestinal microbiota composition, medication and vaccination, physical exercise, stress, appendectomy, breastfeeding, air pollution and heavy metals, as well as exposure of vitamin D to UV (Niewiadomski *et al*., 2016). Smoking has also been proposed to have an influence on the pathogenesis of IBD (Samuelsson, 1976). A sizable proportion of previous research indicates that cigarettes increase the chance of developing Crohn's disease despite protecting against the development of colitis. The mechanism of this phenomenon remains unclear, but can be consequence of changes in the composition of the intestinal microbiota (Biedermann *et al*., 2013).

There is a high probability that the increase in prevalence of IBD seen in the 20th Century is associated with the industrial revolution in Europe and North America. Regional variation has also been observed, insofar as there is large difference in the numbers of cases of autoimmunological diseases, including IBD, between Western and Eastern countries: Based on a review of data from 1920 – 2008, the highest annual incidence of UC was 24.3/100,000 person-years in Europe and 19.2/100,000 person-years in North America compared to 6.3/100,000 person-years in Asia and the Middle East. The highest reported prevalence of UC was observed in Europe (505/100,000 persons) and North America (249/100,000 persons). It has been found that 60 % of documented studies of UC report an increasing number of incidences (Molodecky *et al*., 2012). Other reports indicate that children who have moved from countries with a low IBD incidence to countries with a high incidence have the same probability of developing IBD as the children living in the high-incidence regions (Brobert *et al*., 1992; Li *et al*., 2011).

Hence, environmental factors appear to play a role in the development of IBD, and differences in lifestyle and medical level are reflected in the results of studies. This trend has been attributed to the *Hygienic Hypothesis*, a term first used by Strachan (1989). The Hygienic Hypothesis implies that a lack of immune system actuation in adulthood occurs as a result of maintaining high cleaning standards and avoiding contact with microorganisms during childhood, a potential consequence of which can be the development of a range of immunological disorders, including IBD and allergic diseases. Contact with pathogens is influenced by many factors, including education level, diet, antibiotics and vaccinations, medical and deistical admission, sharing bedrooms or even having pets (Leong *et al*., 2016). If permanent contact with bacteria and viruses is maintained, interaction with multicellular parasites such as nematodes or tapeworms can be eradicated thanks to extensive access to antihelminth drugs and adherence to hygiene rules. In United States schoolchildren, the prevalence of hookworm fell from 65 % in 1910 to fewer than 2 % in 1980 (Kappus *et al*., 1994). The co-evolution of host and parasite resulted in the development of a very complicated mechanism for avoiding the host immunological system, thus increasing the potential for the parasite to

survive and reproduce: Gastrointestinal nematodes cause chronic infection and induce immunosuppression. Such regulation of the immune response by the parasite also offers positive benefits for the host organism: the nematodes control the immunity caused by infection, as well as the responses to various non-nematode antigens (Barthlott *et al*., 2003). However, a strong inflammation response can result in damage to the infected area (Maizels *et al*., 2004). Such deprivation of contact with multicellular parasites observed in Western countries resulting from their high level of hygiene can affect the immunological balance by forcing inequalities in the host-parasite arrangement constructed over millions of years. These phenomena result in the creation of new variants of the *Hygienic Hypothesis*, such as the *Lost Friends Theory* or the *Biome Depletion Theory*. With these theories in mind, it seems like the best option in allergies and autoimmunological disorders treatment is reconstruction of human biome (Bilbo *et al*., 2011).

Immunological Response in the Intestine

The mucosal membrane of the intestine plays a crucial role in the immunological system. The digestive tract is in constant contact with both commensal and pathogenic microorganisms (Macdonald & Monteleone, 2005). The immune system must therefore be able to control symbiotic bacteria and tolerate them, while being able to eradicate pathogens. During colitis, the gut epithelial barrier is dysfunctional (McGuckin *et al*., 2009), and the recognition and response to multiple antigens, commensals or nutrients results in local inflammation of the colon. A hypothesis proposed by Shorter *et al*. (1972) presents that IBD, including colitis, occurs as a result of the establishment of a state of hypersensitivity to the bacterial antigens which are normal components of the intestinal microflora. It is known that other factors further aggravate epithelial-associated dysfunction, which then develops into chronic inflammation of the gastrointestinal tract. Nonetheless, the intestinal microbiota is crucial for the development of IBD and influences the mucosal immune response during active disease.

In healthy patients, the immune system associated with the mucosal gut develops a tolerance to commensal microorganisms and food antigens. three types of Antigen-Presenting Cells (APC), *viz*. dendritic cells (DC), macrophages and B lymphocytes, play a fundamental role in this process (Mann & Li, 2014). DC are able to stimulate primary lymphocyte T cells and differentiate into regulatory T cells (Treg) (Rescigno & Sabatino, 2009), both macrophages and B-cells maintain the survival of Treg, while also secreting interleukin 10 (IL-10) and transforming growth factor β (TGF-β); thus they maintain immune homeostasis and tolerance (Mann & Li, 2014; Hadis *et al*., 2011).

In colitis, antigens emerge from pathogens, food and commensal bacteria which cause intestinal inflammation as a result of the activity of innate immune cells. DC and macrophages secrete proinflammatory cytokines; tumor necrosis factor α (TNF-α), IL-6 and IL-1β. In the adaptive immune response, T helper type 1 cells (Th1) are activated, resulting in strong production of proinflammatory cytokines: interferon γ (IFN-γ), TNF-α and IL-17A (Cader & Kaser, 2013). The existence of a parasite in the host results in Th2 response activation with the production of IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13. In addition, induction of increased populations of basophils, mast cells, eosinophils and alternatively-activated macrophages, together with immunoglobulin G1 (IgG_1), IgG_4 and IgE, are characteristic in cases of multicellular parasite infection (Allen & Maizels, 2011; Maizels *et al*., 2012).

However, the immunoregulatory abilities of helminths inhibit the immunological reaction by the activation of regulatory T lymphocytes (Taylor *et al*., 2012). Higher levels of IL-10 and TGF-β secretion suppress Th1 and Th2 cell activity, consequently protecting both the parasite against expulsion and the host against damage to the tissues caused by the strong inflammatory reaction (Khan & Fallon, 2013). Immunosuppression is specific to helminths but also antigens not associated with infection and hence could be employed in anti-inflammation therapy (Finlay *et al*., 2014).

Colitis Helminth Therapy in Animal Models

Colitis induction methods

Before helminth therapy can be introduced in IBD patients, it is necessary to understand the mechanism of their immunoregulatory abilities. Elliott and colleagues (2000) propose the hypothesis that exposure to helminths can prevent IBD and highlighted the need to formulate a novel chronic intestinal inflammation model for humans. Since then, a few models of colitis induction have been used in rodents to identify a cure for IBD, the main ones being chemically-inducible models, spontaneous models, genetically-modified models and adoptive transfer models (Witrz & Neurath, 2007). Mucosal immune system dysfunction and display of disease manifestation can be achieved in three ways: through defects in epithelial integrity and permeability, deficiency in innate immune cells or by deficiency in adaptive immune cells. These effects can be achieved chemically using dinitrobenzene or trinitrobenzene sulfonic acid (DNBS/TNBS)-inducted colitis (Morampudi *et al*., 2014) or dextran sulfate sodium (DSS)-inducted colitis (Chassaing *et al*., 2015), resulting in mechanical dysfunction of epithelial integrity. Alternatively, IL-10 knockout (IL-10−/−) mice can be used (Keubler *et al*., 2015), which develop spontaneous chronic inflammation in the intestine, i.e. T cell transfer colitis, due to a lack of the main immune regulatory interleukin. In this case, the disease occurs as a result of a deficiency of T regulatory cells (Witrz & Neurath, 2007).

Trematoda

Another issue concerns the variety of species of parasite used in the animal models. Three classes of parasite can be used, namely trematodes, cestodes and nematodes, referred to as helminths. One of the parasite genera belonging to the trematode *Schistosoma* seems to be a promising model for HT. Eliot *et al*. (2003) first published results of TNBS-inducted colitis mice infected by

Schistosoma mansoni eggs which showed attenuation of intestinal inflammation manifestations. A similar effect has been already demonstrated in TNBS-inducted colitis rats after administration of *S. mansoni* larvae (Morels *et al*., 2004). In a different study, infection of mice with *S. mansoni* larvae protected against the manifestation of DSS colitis with macrophage participation (Smith *et al*., 2007). The influence of *S. japonicum* eggs demonstrated a preventive outcome on TNBS-inducted colitis in mice (Zhao *et al*., 2009; Xia *et al*., 2011). In another study, infection with *S. mansoni* larvae of DSS-inducted colitis mice resulted in reduced manifestations and lower levels of Th1 and Th2 cytokines (Bodammer *et al*., 2011) (Table 1).

Cestoda

One of the cestode class, *Hymenolepis diminuta* is also successfully used a model of colitis parasite therapy. Preventive and curative treatment of tapeworm larvae resulted in normalization of colonic ion transport in DSS-inducted colitis mice. No differences in histological or cytokine level were observed (Reardon *et al*., 2001). Hunter *et al*. (2005) demonstrated a reduction of DNBS-inducted colitis symptoms and higher levels of IL-10 and IL-4 in a mouse model. The same team showed increased levels of Th2 and Treg response interleukins in a colitis model induced by oxazolone (Hunter *et al*., 2007). Elsewhere, infection with *H. diminuta* larvae of DNBS-inducted colitis mice resulted in higher levels of Th2 and Treg, and a lower level of Th1 cytokines (Melon *et al*., 2010) (Table 1).

Nematoda

The most promising group of intestinal parasite seems to be the nematodes. *Trichinella spiralis* infection was found to protect mice from developing DNBS-inducted colitis (Khan *et al*. 2002). Another study a decade later reported attenuation of DSS-inducted colitis by *Trichinella papuae* larvae (Adisakwattana *et al*., 2013). Elsewhere, *Trichuris trichiura* eggs restored mucosal barrier functions and reduced overall bacterial attachment to the intestinal mucosa in idiopathic chronic diarrhea in macaque monkeys (Broadhurst *et al*., 2012). The majority of investigations about mechanisms of helminth therapy in human IBD is focused on the gastrointestinal nematode *Heligmosomoides polygyrus*. This parasite of mice, with a simple and short life cycle, is an excellent model of human infection with Necator *americanus* (Monroy & Enriquez, 1992). Both nematodes have been phylogenetically placed in the order *Strongylida* (Gouy *et al*., 2011). Another advantage of using *H. polygyrus* is that its laboratory breeding procedure is uncomplicated. Different tribes of mice react differently to *H. polygyrus* infection, which enables the investigation of the influence of genetic conditioning to the host immunological response. The inflammatory response is reduced during *H. polygyrus* infection, thus demonstrating the suitability of the nematode model in IBD suppression process.

To date there have been numerous reports demonstrating that *H. polygyrus* infection is an effective therapy for colitis. Elliot *et al*. (2004) first demonstrated that *H. polygyrus* larvae can treat colitis in IL-10−/− mice, and later demonstrated suppression of mucosal IL-17 production in the same model (Elliott *et al*., 2008). Infection by *H. polygyrus* larvae in IL10−/− mice with T cell transfer colitis effected induction of CD8+ regulatory cells (Metwali *et al*., 2006). Promising results have been achieved on the same model, showing that DC plays a crucial role in the regulatory immune response in colitis (Hang *et al*., 2010; Blum *et al*., 2012). Studies on TNBS-inducted colitis mice revealed attenuation of the disease with mast cell infiltration following infection by *H. polygyrus* larvae (Setiawan *et al*., 2007; Sutton *et al*., 2008). Our own previous studies found infection with *H. polygyrus* larvae to have a curative effect on DSS-inducted colitis with macrophage infiltration and decreased levels of MOR1, POMC and β-endorphin observed in the colon (Donskow-Łysoniewska *et al*., 2012). Administration of the same larvae to antigen-driven colitis mice also resulted in protection from disease with the induction of $Foxp3^+$ Treg cells (Leung *et al*., 2012).

A treatment effect is not achieved in every model of intestinal inflammation. Investigations in mice with *Citrobacter rodentium*-induced colitis infected with *H. polygyrus* larvae found that DC activation and IL-10 production impaired the host response to *C. rodentium* (Chen *et al*., 2005; Chen *et al*., 2006). Similarly no curative effect was observed in mice with TGF-βRII DN colitis caused by blocking the effect of TGF-β on T cells; the findings showed that TGF-β signaling to T cells can play an essential role in the regulatory abilities of helminths (Ince *et al*., 2009).

All studies clearly show that various species of intestinal parasites have curative and protective effects in animal models. The reports also give an insight into the mechanisms of IBD treatment in humans with helminths. However, these achievements are closely dependent on the method of inducing intestinal inflammation, as well as the choice of parasite species (Table 1).

Therapeutical Potential of Intestinal Helminths

The most common genera of nematodes distributed in human digestive track are *Ascaris*, *Trichuris* (whipworm), *Necator* and *Ancylostoma* (hookworms). Two species of which, *Trichuris suis* and *Necator americanus*, have been investigated in clinical examinations of UC and CD patients. Although *T. suis* is a natural parasite in the caecum and colon of pigs, it can also infect other hosts, including humans; however, the worms can only survive in the human digestive tract for a few weeks (Helmby, 2015).

There is discrepancy in host species and deficiency in inflammatory response for the parasite marked *T. suis* for the most promising nematode for human IBD therapy. The initial results of small clinical studies of IBD treatment with *T. suis* were published in 2003 by Summers *et al*. A group of UC and CD patients received a single oral dose of 2500 live *T. suis* eggs, and were then monitored every 14 days for 12 weeks. A second group of patients received the same administered dosage every 21 days for 28 weeks. After

Table 1. Summary of helminth therapy with live parasites in animal models in IBD.

Author	Model of colitis induction	Parasite class / species	Main outcomes
Eliot *et al*., 2003	TNBS	*Trematoda / Schistosoma mansoni*	Th1 response reduction, Th2 and Treg response induction
Morels *et al*., 2004	TNBS	*Trematoda / S. mansoni*	Th2 response induction
Smith *et al*., 2007	DSS	*Trematoda / S. mansoni*	Macrophage participation
Zhao *et al*., 2009	TNBS	*Trematoda / S. japonicum*	Th1 response reduction
Xia *et al*., 2011	TNBS	*Trematoda / S. japonicum*	Lower intestinal bacterial translocation frequency
Bodammer *et al*., 2011	DSS	*Trematoda / S. mansoni*	Th1 and Th2 response reduction
Reardon *et al*., 2001	DSS	*Cestoda / Hymenolepis diminuta*	No changes in response noticed
Hunter *et al*., 2005	DNBS	*Cestoda / H. diminuta*	Th2 and Treg response induction
Hunter *et al*., 2007	Oxazolone	*Cestoda / H. diminuta*	Th2 and Treg response induction
Melon *et al*., 2010	DNBS	*Cestoda / H. diminuta*	Th2 and Treg response induction and Th1 response reduction
Broadhurst *et al*., 2012	Idiopathic chronic diarrhea in macaques monkeys	*Nematoda / Trichuris trichiura*	Mucosal barrier functions restored and overall bacterial attachment to the intestinal mucosa reduced
Khan *et al*., 2002	DNBS	*Nematoda / Trichinella spiralis*	Th2 response induction
Adisakwattana *et al*., 2013	DSS	*Nematoda / T. papuae*	Th2 response induction and Treg response changes
Elliot *et al*., 2004	IL-10−/−	*Nematoda / Heligmosomoides polygyrys*	Th1 response reduction and Treg response induction
Chen *et al*., 2005	*Citrobacter rodentium*	*Nematoda / H. polygyrys*	STAT 6-mediated mechanism
Chen *et al*., 2006	*Citrobacter rodentium*	*Nematoda / H. polygyrys*	CD11c+ dendritic cells activation and IL-10 production
Metwali *et al*., 2006	IL10−/− T cell transfer	*Nematoda / H. polygyrys*	CD8+ regulatory cells induction
Setiawan *et al*., 2007	TNBS	*Nematoda / H. polygyrys*	Th1 response reduction and Treg response induction
Elliott *et al*., 2008	IL-10−/− mice	*Nematoda / H. polygyrys*	Suppression of mucosal IL-17 production
Sutton *et al*., 2008	TNBS	*Nematoda / H. polygyrys*	mast cells infiltration
Ince *et al*., 2009	TGF-βRII DN	*Nematoda / H. polygyrys*	A role of TGF-β signaling to T cells in regulatory response
Hang *et al*., 2010	IL10−/− T cell transfer	*Nematoda / H. polygyrys*	A role of dendritic cells in regulatory immune response
Blum *et al*., 2012	IL10−/− T cell transfer	*Nematoda / H. polygyrys*	Induction of tolerogenic dendritic cells
Donskow-Łysoniewska *et al*., 2012	DSS	Nematoda / H. polygyrys	Macrophage infiltration and MOR1, POMC, β-endorphin increased levels
Leung *et al*., 2012	Antigen driven	*Nematoda / H. polygyrys*	Induction of $Foxp3^+$ Treg cells

Table 2. Summary of clinical trials of helminth therapy with live parasites in IBD.

Author	Scheme of trial	Results
Summers *et al*., 2003	Single or repeated dose of 2500 live eggs of *T. suis* administered every 3 weeks for 28 weeks to 3 UC and 4 CD patients.	No side effects. Remission noticed on every patient administered with repeated dose.
Summers *et al*., 2005a	Repeated dose of 2500 live eggs of *T. suis* administered every 3 weeks for 24 weeks to 29 CD patients.	No side effects. Remission noticed on 72.4% of patients.
Summers *et al*, 2005b	Repeated dose of 2500 live eggs of *T. suis* administered every 2 weeks for 12 weeks to 54 UC patients.	No side effects. Remission noticed on 43.3% of patients.
Sandorn *et al*, 2013	Single dose of 500, 2500 or 7500 live eggs of *T. suis* administered to 36 CD patients.	Every dose very well tolerated. Quantity of dose has no influence on gastrointestinal tract response.
Croese *et al*, 2006	Single or repeated dose of 25-50 L3 larvae of *N. americanus* administered to 9 CD patients.	Side effects: itching, enteropathy, eosinophilia. Condition of majority of patient's improved.

Abbreviations: UC – ulcerative colitis, CD- Crohne Disease

accurate monitoring, no side effects were noticed and a further repeated dosage resulted in the improvement of all medicated patients (Summers *et al*., 2003). Similarly, in a second clinical trial, a repeated dose of 2500 viable *T. suis* eggs was given to 29 of CD patients every 21 days for 24 weeks, and no adverse reaction was observed. After 24 weeks of therapy, 79.3 % of patients responded to treatment and 72.4 % were in remission, as evaluated based on Crohn's disease activity index (Summers *et al*., 2005a). A similar examination was conducted on 59 UC patients. A dose of 2500 *T. suis* eggs or placebo was given every two weeks for 12 weeks: It was found that 42.3 % of patients who received *T. suis* and 16.7 % of those who received placebo responded, but only 10 % of the first group and 4.2 % of the second displayed remission, with no side effects to induction. The outcome was calculated based on UC disease activity index (Summers *et al*., 2005b).

Almost a decade after helminths were first demonstrated to have promising effects in IBD therapy in humans, Sandborn et al. (2013) demonstrated novel findings concerning the safety and tolerance of various doses of *T. suis* in clinical trials with patients with CD. The patients received one dose of 500, 2500, 7500 *T. suis* eggs or placebo. They were then evaluated for 14 days, and then by telephone interview one, three and six months after receiving the dose. All doses, including the 7500-egg dose, was very well tolerated without any short or long-term adverse reactions (Sandorn *et al*., 2013). In the meantime, *N. americanus* has been proposed as an alternative for *T. suis* and studies have been carried out to investigate tolerance to infection and a number of other practical topics. Humans can be infected with *N. americanus* larvae third-stage (L3) by skin contact with contaminated soil. The adult worms are situated in small intestine of the host and can survive for five years, although expulsion of the parasite is possible with anthelmintic medicines. It is important to note that one consequence of infection is anemia caused by the helminth feeding on blood. In the experiment, CD patients were inoculated with a single or a repeated dose of 25 – 50 infective larvae (L3). Despite a promising remission effect, the presence of the worms yielded a mild itch, painful transient enteropathy and blood eosinophilia (Croese *et al*., 2006).

The outcomes of these studies clearly show the great potential of helminths in IBD therapy, and further study is needed in this area. While just one study has examined *N. americanus*, and side effects were observed, the parasite may still serve as a promising alternative for *T. suis*, especially since *N. americanus* is very well tolerated in CD therapy (Daveson *et al*., 2011; Croese *et al*., 2015). Live *T. suis* and the haematophagous hookworm *N. americanus* have been suggested as effective treatments for IBD, and three clinical trials have been initiated: NCT01040221; NCT01070498; NCT01413243 (Correale, 2014; Ruyssers *et al*., 2008). Nonetheless, a greater understanding of the mechanism by which inflammation is suppressed in the intestine by helminths is essential for further progress in clinical practice (Table 2).

Challenges of Helminth Therapy

During HT, the amelioration of symptoms was only seen when the helminth infection was present; removal of the parasites resulted in the remission of IBD pathology and the inhibition of immunomodulatory response (Fleming *et al*., 2011). Furthermore, many patients feel uneasy about receiving live worms for therapy. In addition, aside from the ethical concerns, there are many practical considerations that may reduce the efficacy of this approach. Nematode L4 larvae invade tissues, and even small numbers of hookworms can induce gastrointestinal or other tissue pain in the early stages of infection; they can also exhibit aberrant migration in the human host and influence the physiology of their respective niches. Live parasite infections result in the induction of danger signals and pro-inflammatory stimuli, thus leading to inflammation. Furthermore, in addition to the desired helminth immunomodulators, the host is exposed to the full spectrum of helminth-derived products including potent antigens, inflammatory stimuli and potentially disease-causing allergens. It is important to note that only

the minimum number of larvae was used in the trial for safety reasons, and so potential clinical benefits may have been lost. In addition, as early infection is characterized by obvious symptoms that will reveal to patients whether they are in a placebo or treatment group, it is very difficult to conduct trials by incorporating proper placebo controls. Furthermore, helminths can influence drug efficacy by modulating the host immune response, and colonization may worsen other pathogenic infections in immunocompromised hosts (Correale, 2014).

Treatment with living nematodes therefore has clear disadvantages, and in order to survive for a long time in an adverse and aggressive environment, the nematodes may modify host-cell homeostasis and increase susceptibility to oncogenic transformation by secreting several soluble factors that interact with host cells (Packham & Stevenson, 2005; Donskow *et al*., 2011; Donskow-Łysoniewska *et al*., 2013b). The factors secreted by helminths could be involved in neoplasma promotion and progression. *Schistosoma haematobium, Spiromera mansonoides, Taenia taeniaformis,* and *T. solium*, all have significant tumor-promoting activity (Herrera & Ostrosky-Wegman, 2001). Excretory-secretory (ES) products from the small intestine nematodes *Trichostrongylus vitrinus, T. colubriformis, Cooperia curticei, Nematodirus battus* and the abomasal nematode *Teladorsagia circumcincta* have all been shown to produce over-proliferation in normal intestinal epithelial cells and/or cell lines (Huby *et al*., 1995). Additionally, our study indicated that in live nematode therapy of colitis, the changes in the small intestinal *milieu* promote intestinal nematode larval adaptation and improve worm growth. The plasticity of the nematode proteome is a consequence of evolutionary adaptation which benefits the host by inhibiting inflammatory disease and also the parasite by increasing its survival (Donskow-Łysoniewska *et al*., 2013a) (Table 3).

Other Perspectives

Even though the mechanism of disease prevention is unknown, HT seems to be the most effective therapy of IBD currently proposed. As HT has its disadvantages, an important aim of HDPT research is to characterize the key molecules responsible for immunomodulation for use as drugs to control inflammation and autoimmune diseases such as IBD. For this reason, a number of international studies have attempted to identify the immune-active components of helminths. Some filarial nematode proteins such as cystatin (AvCystation) have been shown to prevent asthma and colitis by induction of IL-10 production by macrophages in animal models of the disease (Schnoeller *et al*., 2008). A filarial-derived phosphorylcholine product (ES-62) of *Acanthocheilonema vitae* modulates dendritic cell and macrophage activity in a toll-like receptor 4 (TLR-4) dependent manner and attenuates the symptoms of collagen-induced arthritis (CIA), aryl hydrocarbon receptor (AHR) knockout, and DSS-induced colitis (Goodridge *et al*., 2005). The recombinant 53kDa protein from *T. spiralis* prevents experimental colitis in mice and upregulates Th2 and regulatory cytokines while downregulating some Th1 cytokines (Du *et al*., 2011).

However, studies of the potential therapeutic use of single immune-active components isolated from nematode products is not as meaningful as previously suggested. The live nematodes express and secrete copious quantities of antigens into host tissues with different immunomodulatory properties, and the immunomodulatory effects, presumably intended for self-protection, must be multiple and complex. These mixtures of proteins, peptides, glycans and lipids might help the worm to survive in a number of ways, minimizing inflammatory processes or interfering with them, and selectively skewing the phenotype of the immune response generated (Mulyenna *et al*., 2009).

The protective immune responses to native antigens have been difficult to replicate based on recombinant antigens produced in most popular artificial expression systems, such as bacteria and yeast, as these usually have an incorrect conformation and the lack post-translational modifications of the recombinant molecule. Increasingly, post-translational modifications such as those including phosphocholine (PC) and various glycans are being recognized as the active components of many immunomodulatory components of helminths (ICHs), particularly in interactions with the host (Prasanphanich *et al*., 2013; Hokke *et al*., 2007). Furthermore, the use of bare single-defined immunomodulatory products as therapeutics is doomed to failure as such products can be neutralized and rendered ineffective by the host immune response.

In addition, the use of helminth excretory secretory (ES) products does not solve the problem. These represent up to 30 % of the proteome of an organism, and proteomic studies have found them to

Table 3. Cons and pros of using helminth therapy with live parasites.

CONS	PROS
▪ Exposure to full spectrum of helminth products ▪ Tissue invasion by helminths ▪ Ethical aspect ▪ Symptoms re-emergence after parasites removal ▪ Tumor promoting activity ▪ Better adaptation of worms in colitis environment ▪ Difficulty in proper placebo controls use ▪ Proinflammatory activity of live worms	▪ Strong immunogenic properties of live parasite ▪ Better than any therapy currently available ▪ An introduction to more extensive research using molecules with immunomodulatory properties ▪ Less expensive method

Table 4. Comparison of effectiveness and safety of various ways of helminth therapy.

EFFECTIVENESS			SAFETY
Live parasites	**ES products**	**Single parasite compounds**	**Synthetized proteins based on parasite compounds**

be highly distinct from somatic extracts (McSorley *et al*., 2013). However, the range of secretory products is wide and varied, comprising a complex mixture of many different substances with particular biological functions which are secreted from cells or glands, as well as various unnecessary metabolic products released from the body. Hence, it is difficult to determine the precise application of ES products from parasitic helminths: the analysis of the smaller molecules among ES products can be confounded by protein breakdown products and media components used for *in vitro* culture of nematodes, including amino acids with immunomodulatory properties in their own right. In addition, due to the low concentrations of protein caused by high dilutions of cultivation media, ES can often be contaminated by normally non-secreted proteins following nematode cell lysis and death (Smith *et al*., 2009).

Therefore, the somatic extract might be extremely useful in the development of intervention strategies for inflammatory reactions, especially since the immunomodulatory potency of helminths appears to be largely achieved by their surface glycoproteins (Erb, 2009). As the immune regulation induced during parasitic infection is complex and cannot be generated by single recombinant factors, and therapy with live nematodes could produce a severe infection, it appears essential to devise other modes of treatment with nematode products, acting as a silver bullet, particularly since a fully-effective treatment for autoimmune and allergic disease remains unknown (Table 4).

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High IL-1α production was induced in the WBN/Kob-*Lepr*^{fa} type 2 diabetes mellitus rat model and inhibited by *Syphacia muris* infection

M. OKAMOTO[1, §, *], R. ITO[1, §], K. TAIRA[2], T. IKEDA[1]

[1]Laboratory of Veterinary Immunology, Department of Veterinary Medicine, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan, E-mail: *m-okamoto@azabu-u.ac.jp*;
[2]Laboratory of Parasitology, Department of Veterinary Medicine, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

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Summary

The novel WBN/Kob-*Lepr*^{fa} (*fa/fa*) congenic rat strain is considered a useful rat model of type 2 diabetes mellitus (T2DM). Accumulating findings suggest that low-grade inflammation is a causative factor in T2DM and that circulating levels of inflammatory cytokines are associated with insulin resistance. However, inflammatory cytokine profiles and their correlations with T2DM development/progression in *fa/fa* rats have not been studied. In this study, we found that the *fa/fa* rats had considerably high plasma levels of interleukin (IL)-1α. Abundant cecal IL-1α mRNA expression and cecal inflammation with infiltrating IL-1α-producing macrophages was observed in *fa/fa* rats. Bone marrow derived macrophages from *fa/fa* rats expressed high levels of IL-1α upon lipopolysaccharide stimulation. Furthermore, *Syphacia muris* infection, which delays the onset of T2DM, reduced both plasma and cecal IL-1α levels in *fa/fa* rats. These results suggest that macrophage infiltration and IL-1α secretion comprise an important part of T2DM development and that *S. muris* infection inhibits pro-inflammatory cytokine expression in *fa/fa* rats.
Keywords: IL-1α; macrophage; *Syphacia muris*; type 2 diabetes mellitus; WBN/Kob-*Lepr*^{fa}

Introduction

Type 2 diabetes mellitus (T2DM) is a major international health problem with an increasing global prevalence (Farag *et al.*, 2011, Zimmet *et al.*, 2001). Approximately 95 % of patients with diabetes mellitus suffer from T2DM, which is characterized by insulin resistance and pancreatic β-cell dysfunction. Recent studies have demonstrated that chronic low-grade inflammation with elevated levels of pro-inflammatory cytokines contributes to the onset and progression of T2DM (Donath *et al.*, 2011). For example, interleukin (IL)-1β, a representative pro-inflammatory cytokine, is produced via the Nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome pathway following activation by free fatty acids (Wen *et al.*, 2011). IL-1β leads macrophage recruitment and islet β-cell death by inducing upregulation of the pro-apoptotic receptor Fas (Maedler *et al.*, 2002). Anti-inflammatory approaches that target specific pro-inflammatory cytokine pathways are expected to be efficacious in the treatment or prevention of T2DM.

Animal models of T2DM are needed to elucidate pathogenesis and conduct pharmacological studies of new candidates for therapeutic or preventive options. The WBN/Kob-*Lepr*^{fa} (*fa/fa*) rat is a new congenic strain established by introducing the leptin receptor fatty gene (*Lpr*^{fa}), a spontaneous mutation derived from Zucker-fatty rats that develop obesity and hyperinsulinemia consequent to leptin receptor dysfunction (Zucker, 1965; Chua *et al.*, 1996), into the parental WBN/Kob rat genome (Akimoto *et al.*, 2008). Previous reports revealed that *fa/fa* rats develop hyperglycemia at 9 weeks of age, evidenced by impaired glucose tolerance, insulin resistance, and dyslipidemia in a manner similar to human T2DM8 (Akimoto *et al.*, 2008; Akimoto *et al.*, 2012; Kaji *et al.*, 2012; Okuno

* – corresponding author
§ – These authors contributed equally to this work.

et al., 2013; Nagakubo *et al.*, 2014), thus suggesting that the *fa/fa* rat is useful as a new model of T2DM.

In a previous study, we demonstrated that infection with *Syphacia* (*S.) muris*, a nematode that targets rats, delayed the onset of hyperglycemia in *fa/fa* rats (Taira *et al.*, 2015). Furthermore, a global transcriptomic next-generation sequencing analysis of *S. muris* suggested that some genes expressed in *S. muris* might modify the host immune response; these genes include a gene involved in the regulation of macrophage activation via the Fc receptor signaling pathway (Okamoto *et al.*, 2015). Those studies suggested that *S. muris* infection might regulate the local production of cytokines and chemokines that promote the development of T2DM. However, the inflammatory cytokine profiles and their contributions to the development and progression of T2DM have not been investigated in *fa/fa* rats. The aim of present study, therefore, was to evaluate cytokine/chemokine profiles in *fa/fa* rats and compare them with profiles of control Wistar rats or *S. muris*-infected *fa/fa* rats. In addition, cells, organs or tissues, and/or tissues that produce inflammatory cytokines in *fa/fa* rats were also investigated.

Materials and Methods

Animals

Male WBN/Kob-*Lepr*^{fa} (*fa/fa*) and Wistar rats (control) were obtained from SLC Inc. (Shizuoka, Japan). All rats were maintained under conventional conditions with a 12 h on/off light cycle, commercial diet, and water *ad libitum*. All animal studies were approved by the Azabu University Animal Research Ethics Committee (approval No. 150303-3). All animal procedures were performed under the experimental animal guidelines of Azabu University. Blood glucose levels of *fa/fa* rats were measured weekly.

Parasites and infection

S. muris infection was performed as described previously (Taira *et al.*, 2015). Briefly, *S. muris* eggs were harvested from the uteruses of female worms obtained from the colons and rectums of experimentally infected male Wistar rats (Stahl, 1961). At 5 weeks of age, *fa/fa* rats were inoculated once via stomach tubes with either approximately 100 eggs/ml/rat or 1 ml/rat of distilled water (for uninfected group). *S. muris* infection was confirmed using cellophane tape to detect pinworm eggs in the perianal regions of rats.

Blood sampling

Blood samples were collected from the tail vein, and plasma samples were obtained by centrifuging heparinized blood. In some experiments we used Wistar rats' plasma purchased from SLC Inc. as a "control."

Cytokine and chemokine array

To measure plasma cytokine and chemokine levels, pooled plasma from 9 – 10-week-old *fa/fa* rats, *S. muris*-infected *fa/fa* rats, and control Wistar rats plasma (SLC Inc.) were used. Cytokine profiles were analyzed using the Proteome Profiler™ Rat Cytokine Array Panel A (R&D Systems, Wiesbaden, Germany), which can identify 29 cytokines (Fig. 1A). All experiments were performed according to the manufacturer's instructions. Spots were visualized using Immobilon Western (Merck Millipore, Massachusetts, USA) and captured with an ImageQuant LAS-4000 (GE Healthcare, Buckinghamshire, UK). ImageQuant TL software (GE Healthcare) was used for the data analysis. Signals were normalized using internal controls included on the array.

Enzyme-linked immunosorbent assay (ELISA)

IL-1α levels in plasma or bone marrow-derived macrophage (BMDM) culture supernatant were quantified using a Rat IL-1α Platinum ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (RT-qPCR)

RT-qPCR of IL-1α was performed as described previously (Okamoto *et al.*, 2009), using RNA from ceca isolated from 10-week-old *fa/fa* and *S. muris*-infected *fa/fa* rats and Wistar rats. Total RNA was extracted from each cecum using an RNeasy Mini kit (QIAGEN, Hilden, Germany). Single-stranded cDNA was synthesized from total RNA using the RT2 First Strand Kit (QIAGEN). IL-1α mRNA expression was determined by RT-qPCR with the RT2 SYBR Premix and primer sets for IL-1α (ref.seq. NM_017019.1) and β-actin (ref.seq. NM_031144.2) or 18s RNA (ref.seq. X01117.1); these reagents were purchased from QIAGEN and used according to the manufacturer's instructions. Relative target gene quantification was calculated according to the comparative CT method.

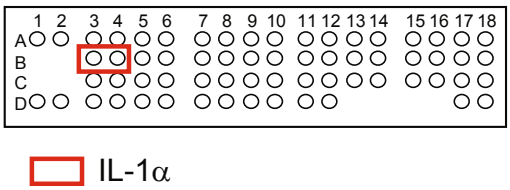
Histologic analysis

Cecal tissues were isolated from 12−16-week-old *fa/fa* rats and Wistar rats. Tissues were fixed in a 10 % neutral buffered formalin solution and embedded in paraffin blocks or, for frozen sections, in OCT compound (Sakura FineTek, Tokyo, Japan) after snap-freezing in liquid nitrogen as described previously (Nagakubo *et al.*, 2014; Aihara *et al.*, 2015). Histologic sections (4-μm-thick) were stained with hematoxylin and eosin (HE).

Immunofluorescence analysis

To detect IL-1α expression in the cecum, frozen cecal sections were incubated with a rabbit anti-IL-1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Alexa Flour 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) was used for secondary detection. To detect macrophages in the cecum, frozen sections were incubated with mouse anti-CD68 (Serotec, Oxford, UK). Alexa Flour 568-conjugated goat anti-mouse IgG (Invitrogen) was used for secondary detection. Sections were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Stained sections were examined and imaged using a fluorescence microscope (FSX100; Olympus, Tokyo, Japan).

A



coordinate	target	coordinate	target
A1, A2	control	C3, C4	IL-13
A3, A4	CINC-1	C5, C6	IL-17
A5, A6	CINC-2α/β	C7, C8	CXCL10
A7, A8	CINC-3	C9, C10	LIX
A9, A10	CNTF	C11, C12	L-Selectin
A11, A12	CX3CL1	C13, C14	CXCL9
A13, A14	GM-CSF	C15, C16	CCL3
A15, A16	sICAM-1	C17, C18	CCL20
A17, A18	IFN-γ	D1, D2	control
B3, B4	IL-1α	D3, D4	CCL5
B5, B6	IL-1β	D5, D6	CXCL7
B7, B8	IL-1ra	D7, D8	TIMP-1
B9, B10	IL-2	D9, D10	TNF-α
B11, B12	IL-3	D11, D12	VEGF
B13, B14	IL-4	D17, D18	negative control
B15, B16	IL-6		
B17, B18	IL-10		

B

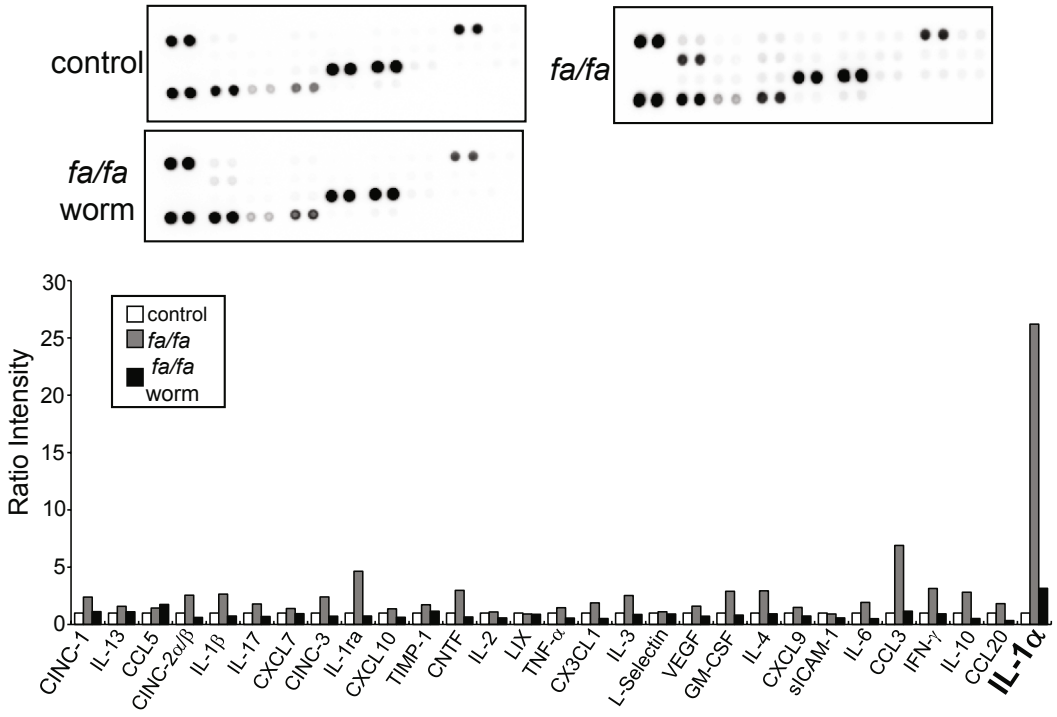


Fig. 1. Profile of circulating cytokines and chemokines in rats according to an array panel
(A) Rat cytokine/chemokine array panel coordinates. The IL-1α coordinate is indicated by a box with red line.
(B) Plasma cytokines and chemokines from Wistar rats (control), *fa/fa* rats (*fa/fa*), and *Syphacia muris*-infected *fa/fa* rats (*fa/fa* worm) were measured using a rat cytokine/chemokine array panel. The signal intensity at each spot was normalized using internal controls included on the array, and relative levels of each cytokine/chemokine are presented as a graph.

Macrophage differentiation and stimulation

BMDMs were generated from bone marrow cells collected from the femurs of 8-week- old *fa/fa* rats and Wistar rats, followed by *in vitro* differentiation in high-glucose Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10 % heat-inactivated fetal bovine serum, 100U/ml penicillin/streptomycin, 2mM L-glutamine, and 10 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF; Peprotech, Rocky Hill, NJ, USA). Cells were maintained in 5 % CO2 at 37˚C. On days 3 − 4, BMDMs were counted and seeded into 24-well plates (5 x 10^5 cells/well). Cells were incubated overnight and then stimulated with 1 μg/ml of bacterial lipopolysaccharide (LPS, *E.Coli* O55:B5; Sigma-Aldrich, St. Louis, MO, USA). Cells and supernatants were harvested 24 hours after LPS stimulation for gene expression studies. IL-1α RT-qPCR and ELISA analyses were carried out as described above.

Statistical analysis

Data are presented as means ± standard errors (SEs). The analysis involved a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Student's t test for paired observations was used for the statistical comparison of BMDC IL-1α expression. Results were considered statistically significant at a p value <0.05.

Results

Array panel comparison of inflammatory cytokines and chemokines

We first valuated plasma cytokine/chemokine profiles of WBN/Kob-*Lepr^{fa}* (*fa/fa*) rats that developed diabetes, characterized by high plasma glucose levels at 9 weeks of age, using the cytokine/chemokine array panel (Fig.1A). As shown in Fig. 1B, the array analysis revealed very low levels of cytokines and chemokines in the plasma of control rats. In contrast, the plasma levels of most cytokines and chemokines were at least 2-fold higher in *fa/fa* rats than in control rats, although the signal at each spot was relatively weak (Fig. 1B). Among these elevated cytokines, we detected an extremely high level of IL-1α in plasma from *fa/fa* rats (Fig. 1B). The levels of IL-1α was higher than that of IL-1β, another IL-1 family cytokine that has been reported to associate with chronic inflammation in obesity (Fig. 1B).

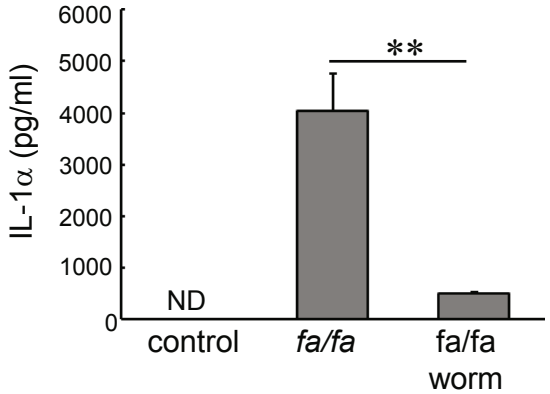


Fig. 2. Plasma IL-1α protein levels in control, fa/fa and *Syphacia* (*S.*) *muris*-infected *fa/fa* rats. Plasma IL-1α levels in Wistar rats (control), *fa/fa* rats (*fa/fa*), and *S. muris*-infected *fa/fa* rats (*fa/fa* worm) were measured by enzyme-linked immunosorbent assay. Data are presented as means ± standard errors (n = 4). **: p <0.01. ND = not detected.

fa/fa rats exhibit elevated plasma levels of IL-1α

We next examined the amount of IL-1α in plasma from control and *fa/fa* rats by ELISA. IL-1α was not detected in plasma from control

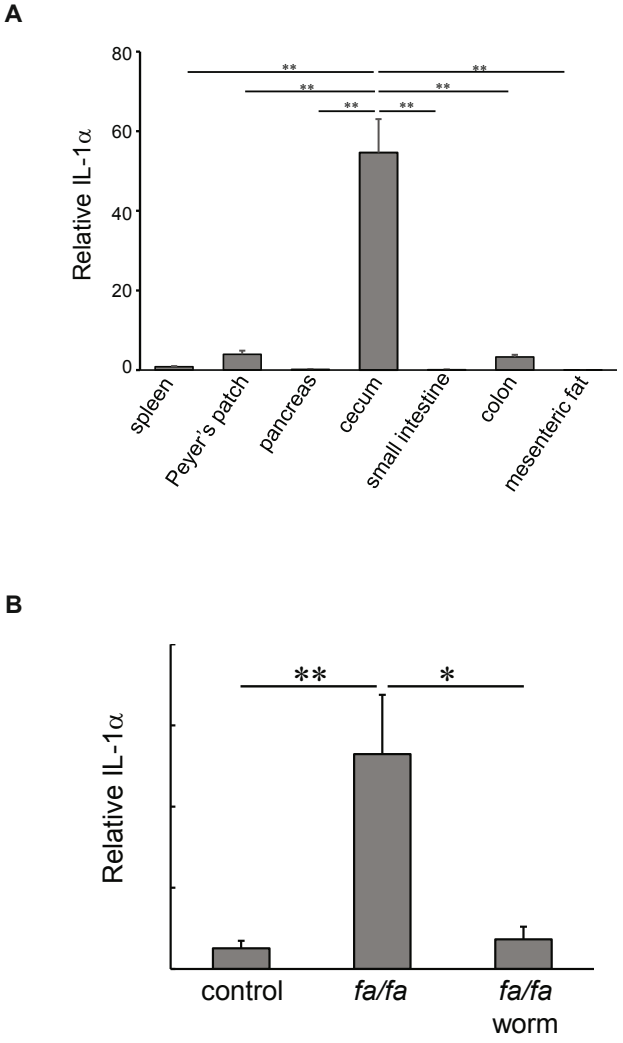


Fig. 3. Relative IL-1α mRNA levels in the ceca of control, fa/fa and *Syphacia* (*S.*) *muris*-infected fa/fa rats. (A) IL-1α mRNA expression levels among organs and tissues (spleen, Peyer's patch, pancreas, cecum, intestine, colon, mesenteric fat) were measured by real-time quantitative PCR. (B) IL-1α mRNA expression levels in the ceca of Wistar rats (control), *fa/fa* rats (*fa/fa*), and *S. muris*-infected *fa/fa* rats (*fa/fa* worm) were measured by real-time quantitative PCR.
Data are expressed as means ± standard errors (n = 4). *: p <0.05, **: p <0.01.

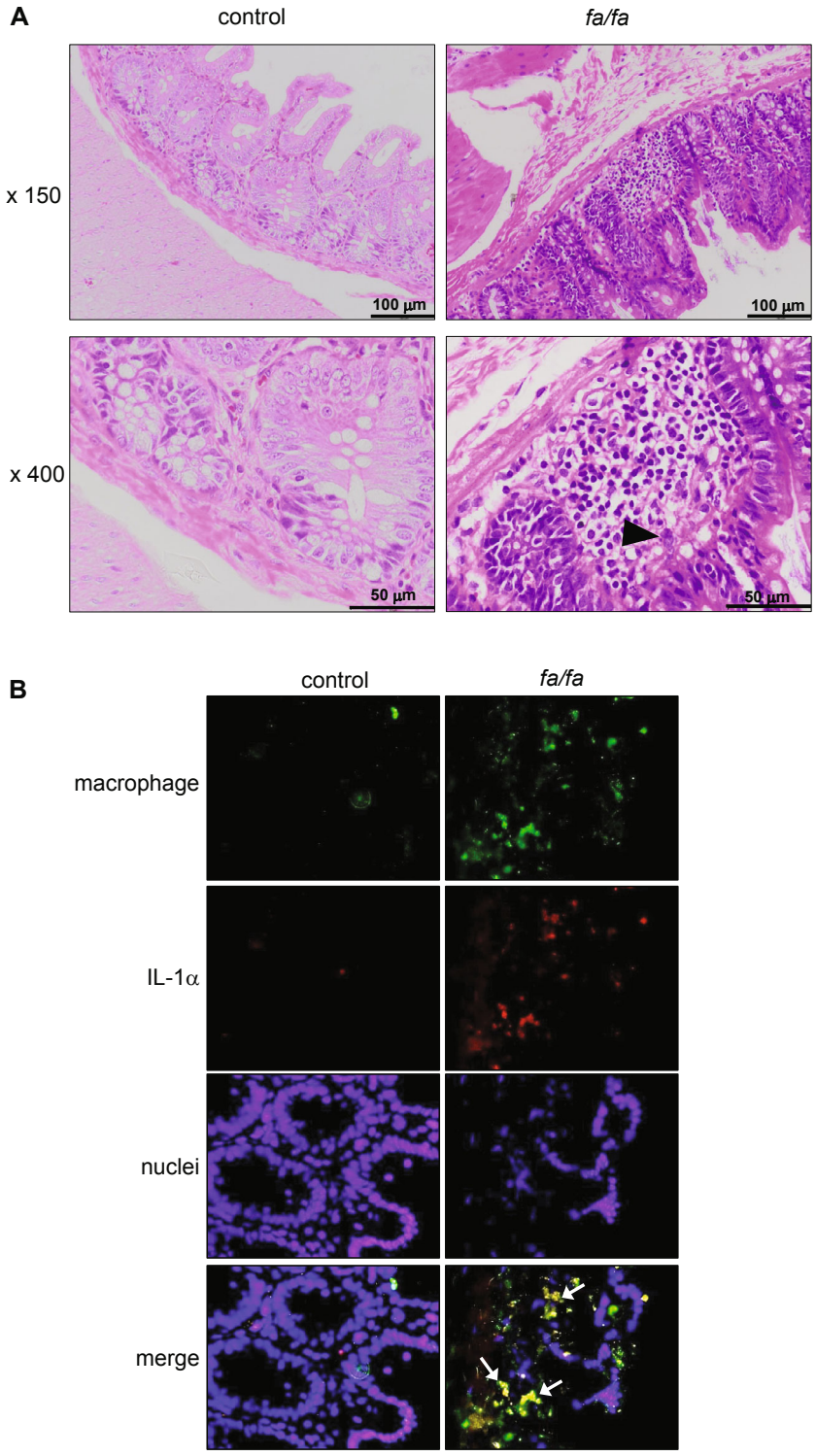


Fig. 4. Cecal inflammation with IL-1α producing-macrophage infiltration observed in *fa/fa* rats.
(A) Representative hematoxylin and eosin-stained cecal sections from control and *fa/fa* rats. Low-powered (x150, top) and high-powered (x400, bottom) images of ceca of Wistar rats (control) and *fa/fa* rats (*fa/fa*). Mucosal infiltrates of lymphoid cells and macrophages (arrowhead) are visible in the ceca of *fa/fa* rats. (B) Representative immunohistochemical images of F4/80 and IL-1α. Cecal sections of Wistar rats (control) or fa/fa rats (fa/fa) were stained with antibodies against macrophage marker F4/80 (green) and IL-1α (red). Nuclei were stained with DAPI (blue). Images were captured at 150× magnification. Macrophages expressing IL-1α (arrow) are visible in the ceca of *fa/fa* rats.

rats (Fig. 2). On the other hand, the amount of plasma IL-1α in *fa/fa* rats was extremely elevated in comparison with control rats (Fig. 2); in other words, these results were similar to those obtained from the cytokine/chemokine array panel (Fig. 1B). These findings, taken together with the results in Fig. 1B, demonstrate that the pro-inflammatory cytokine IL-1α is the main cytokine produced in *fa/fa* rats that have developed diabetes.

Ceca from fa/fa rats express high levels of IL-1α mRNA

We used RT-qPCR to investigate IL-1α expression in various organs of *fa/fa* rats in an attempt to identify the sources of the high levels of circulating IL-1α. Total RNA was extracted from various organs or tissues (including spleen, Peyer's patch, pancreas, cecum, intestine, colon, mesenteric fat) with suspected involvement in the production of inflammatory cytokines associated with the onset of T2DM. Among the organs and tissues analyzed in *fa/fa* rats, the highest levels of IL-1α were found in the cecum (Fig. 3A). As shown in Fig. 3B, the IL-1α mRNA expression levels in the ceca of control rats were very low, whereas those of *fa/fa* rats were significantly increased. IL-1α expression was not detected in the spleen or pancreas in *fa/fa* rats (Fig. 3A). These results suggest that the cecum is one of the main sources of the high levels of plasma IL-1α observed in *fa/fa* rats.

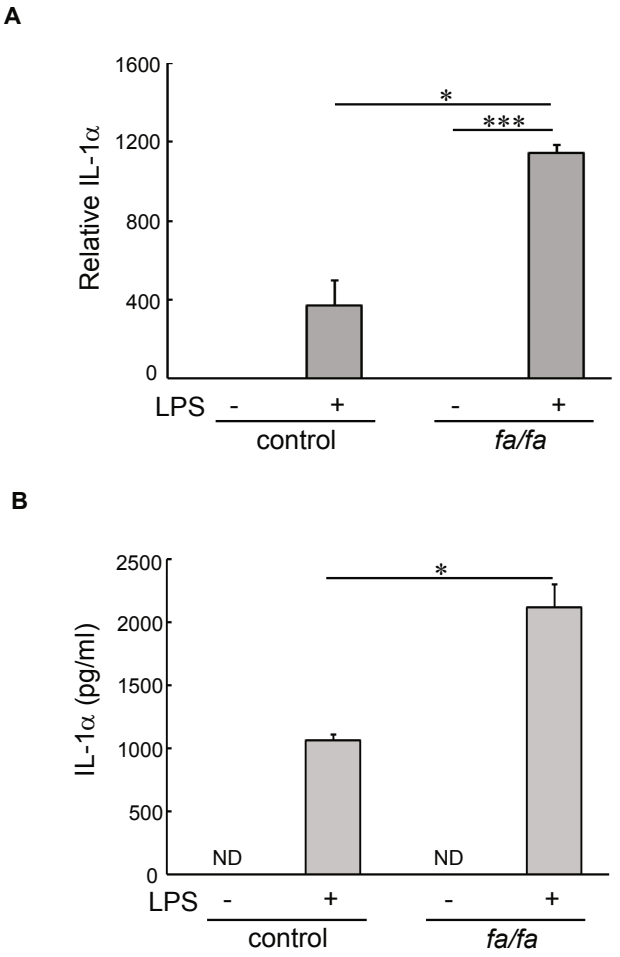


Fig. 5. Levels of IL-1α produced by bone marrow-derived macrophages (BMDMs) from control and *fa/fa* rats. BMDMs from Wistar rats (control) or *fa/fa* rats (*fa/fa*) were unstimulated or stimulated with LPS (1 μg/ml). (A) IL-1α mRNA expression levels were determined by real-time quantitative PCR. (B) IL-1α protein levels in supernatants were measured by enzyme-linked immunosorbent assay. Data are expressed as means ± standard errors (n = 3). ∗: p <0.05, ∗∗: p <0.01, ∗∗∗: p <0.001.

fa/fa rats exhibit cecal inflammation with macrophage infiltration

IL-1α has recently been reported to associate with sterile inflammation (Freigang *et al.*, 2013). Because high levels of IL-1α were expressed in the ceca of *fa/fa* rats with diabetes, a histological analysis of the ceca from *fa/fa* rats was conducted to examine the presence of inflammation. These histological findings are shown in Fig. 4A. Pathological cecal changes were not observed in control rats. In contrast, the ceca of *fa/fa* rats exhibited inflammatory cell infiltration into the lamina propria (Fig. 4A). Although focal inflammatory infiltrates in the cecal mucosa were mainly composed of lymphocytes, macrophage infiltration was also observed (Fig. 4A, arrowhead). We further subjected cecal sections from control or *fa/fa* rats with diabetes to an immunohistochemical analysis with antibodies against the macrophage marker F4/80 and against the C-terminal of IL-1α. As shown in Fig. 4B, *fa/fa* rats contained increased numbers of F4/80-positive macrophages in the cecal mucosa, compared with control rats. Furthermore, IL-1α protein-producing macrophages were detected in cecal sections of *fa/fa* rats, but not of control rats (arrows in Fig. 4B). These results suggest that macrophage infiltration and consequent enhanced IL-1α production might induce cecal inflammation in *fa/fa* rats.

Increased IL-1α production from fa/fa BMDMs upon LPS stimulation

Thus far, our results suggested that pro-inflammatory responses of macrophages are enhanced in *fa/fa* rats relative to control rats. To evaluate this further, we stimulated BMDMs from *fa/fa* or control rats with bacterial lipopolysaccharide (LPS), which induces proinflammatory cytokine production through toll-like receptor (TLR) 4 activation (Jin *et al.*, 2008; Kawai *et al.*, 2011). We observed no differences in macrophage differentiation rates, which were determined using a macrophage-specific antibody against F4/80 (Santa Cruz Biotechnology), between *fa/fa* or control rats. Very low levels of IL-1α mRNA were detected in the absence of stimulation, and IL-1α expression was induced by LPS stimulation in BMDCs from both genotypes (Fig. 6A). However, the IL-1α mRNA expression levels in *fa/fa* BMDCs upon stimulation were significantly higher than those in control BMDCs (Fig. 6A). We also measured the amount of IL-1α protein in culture supernatants; as shown in Fig. 6B, culture supernatants from LPS-stimulated *fa/fa* BMDCs contained much higher amounts of IL-1α than did those from stimulated control BMDCs. These findings indicate that macrophages derived from *fa/fa* rats exhibit enhanced responsiveness to pro-inflammatory stimuli.

fa/fa rats infected with S. muris decreases plasma cytokine/chemokine levels and especially prominent elevated level of IL-1a in plasma and cecum

In a previous study, we demonstrated that infection with *S. muris* delayed the onset of T2DM in *fa/fa rats*, and suggested that this nematode infection might regulate the local production of cytokines/chemokines production that promote T2DM development (Taira *et al.*, 2015). To examine the effect of *S. muris* infection on cytokine/chemokine production in *fa/fa* rats, we first investigated changes in plasma cytokine/chemokine levels in *fa/fa* and *S. muris*-infected *fa/fa* rats. An array analysis of circulating cytokines/chemokines revealed that when compared with uninfected *fa/fa* rats, the plasma levels of most cytokines, including IL-1β and chemokines were decreased in *S. muris*-infected *fa/fa* rats to the levels similar to those detected in control rats (Fig. 1B). Furthermore, we found that the levels of IL-1α which were extremely high in *fa/fa* rats, were also dramatically reduced in *S. muris*-infected *fa/fa* rats (Fig. 1B). ELISA analysis also demonstrated a remarkable decrease in the plasma IL-1α concentration in *fa/fa* rats infected with *S. muris*, compared with uninfected rats (Fig. 2). These results indicate that *S. muris* infection decreases the levels of circulating IL-1α in *fa/fa* rats. We further analyzed IL-1α mRNA levels by RT-qPCR to determine whether *S. muris* infection would affect IL-1α expression in the ceca of *fa/fa* rats. As shown in Fig. 3, the relative expression level of IL-1α mRNA in the ceca of *S. muris*-infected *fa/fa* rats was significantly reduced when compared with that observed in uninfected rats, and was similar to the level detected in control rats. These findings suggest that *S. muris* infection inhibits the expression of cecal IL-1α in *fa/fa* rats with diabetes; in addition, this inhibition might delay the onset of T2DM by reducing cecal inflammation.

Discussion

Currently, chronic inflammation is well accepted as a critical factor in the onset and progression of T2DM (Donath *et al.*, 2011). Chronic inflammation is characterized by an increase in circulating inflammatory cytokines (Rodriguez-Hernandez *et al.*, 2013). In this study, we observed increased plasma levels of cytokines/chemokines in *fa/fa* rats with diabetes relative to those in age-matched control rats (Fig.1B). Likely, this increase in circulating cytokines results from pancreatic inflammation, as this strain naturally exhibits pancreatitis at 7 – 9 weeks of age (Akimoto *et al.*, 2012). We detected high plasma levels of IL-1α rather than IL-1β which is known to associate with T2DM (Fig.1B and Fig. 2). Although the circulating IL-1β level was lower than the IL-1α level in *fa/fa* rats, it remained almost 2.6-fold higher than the plasma IL-1β level in control rats (Fig. 1B). This result suggests that IL-1β might also be involved in the onset of diabetes in *fa/fa* rats.

Aberrant IL-1α signaling is involved in a wide range of inflammatory diseases, including arthritis, atherosclerosis, and diabetes (Kamari *et al.*, 2011; Kamari *et al.*, 2007; Banerjee *et al.*, 2012; Rider *et al.*, 2013). However, when compared with IL-1β, little is known about the contribution of IL-1α to the development of T2DM. Recently, Freigang *et al.*, reported that IL-1α was selectively produced and secreted from macrophages upon stimulation via fatty acids such as oleic acid, leading to atherogenesis via vascular inflammation (Freigang *et al.*, 2013; Spears *et al.*, 2013). In our present study, cecal IL-1α expression was significantly higher in *fa/fa* rats with diabetes than in control rats (Fig. 3). Our data demonstrated elevated macrophage infiltration into the ceca of *fa/fa* rats, compared with control rats; in addition, the cecal macrophages in *fa/fa* rats expressed high levels of IL-1α (Fig. 4). Furthermore, IL-1α production in BMDMs derived from *fa/fa* rats in response to innate immune stimulation was significantly higher than in control BMDMs (Fig. 5). Recently, Jourdan *et al.*, reported that endocannabinoid-activated infiltrating macrophages led to islet β-cell loss in a rat model of T2DM (Jourdan *et al.*, 2013), suggesting that infiltrating macrophages and the consequent inflammatory cytokines play an important role in the onset of T2DM. Although the molecular mechanisms that control the increased production of IL-1α from macrophages of *fa/fa* rats remains unclear, previous report noted that activation of the absent in melanoma 2 (AIM2) inflammasome led to the release of higher levels of IL-1α but not IL-1β via calpain activation (Sorrentino *et al.*, 2015). Likewise, our results suggest that in *fa/fa* rats, macrophages are activated by stimuli such as free fatty acids from adipocytes or islet amyloid polypeptides, thus inducing IL-1α expression and secretion along with migration to the cecum.

We previously demonstrated that *S. muris* infection decreases plasma glucose level and delays the onset of T2DM in male *fa/fa* rats (Taira *et al.*, 2015). In the present study, *S. muris*-infected *fa/fa* rats exhibited decreased levels of circulating cytokines, including IL-1α and IL-1β and chemokines (Fig. 1B and Fig. 2). Furthermore, *S. muris*-infected *fa/fa* rats exhibited reduced cecal IL-1α expression (Fig. 3B). These results suggest that *S. muris* could regulate the production of IL-1α in the cecum, the site of parasitization for this nematode. Our previous study revealed that *S. muris* expresses mRNAs for Fc receptor signaling proteins that might control macrophage activation (Okamoto *et al.*, 2015). This report and our present study suggest that cecal macrophages are a candidate target for *S. muris*-mediated suppression of inflammatory reactions. We have not yet examined whether *S. muris* directly interacts with and regulates macrophage activation. In addition, it remains unclear whether a correlation exists between the cecal expression of IL-1α and pancreatitis. Future studies intended to identify the involvement of IL-1α or IL-1α-producing macrophages in pancreatitis and the onset of T2DM and to elucidate the molecular mechanism underlying the *S. muris*-mediated inhibition of inflammatory cytokine production will be indispensable.

Conflict of interest

None of the authors have any conflict of interests.

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Complementary effect of *Capparis spinosa* L. and silymarin with/without praziquantel on mice experimentally infected with *Schistosoma mansoni*

S. S. EL-HAWARY[1], K. F. TAHA[2], F. N. KIRILLOS[1], A. A. DAHAB[3], A. A. EL-MAHIS[2], S. H. EL-SAYED[4, 5]*

[1]Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt; [2]Applied Research Center of Medicinal Plants, National Organization of Drug Control and Research, Cairo, Egypt; [3]Department of Medicinal and Aromatic Plants, Horticulture Research Institute, ARC, Cairo, Egypt; [4]Medical Parasitology Department, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt; [5]Medical Parasitology Department, Faculty of Medicne, Helwan University, Cairo, Egypt,
*E-mail: *dr_sh_helmy@yahoo.com*, *shaimaahelmy13@gmail.com*

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Summary

Schistosomiasis remains to be the most common fibrotic disease resulting from inflammation and deposition of scar tissue around trapped parasitic eggs in the liver. Though chemotherapy eradicates matured worms efficiently and prevents the accumulation of *schistosome* eggs, fewer effective drugs are directed to reverse the present hepatic fibrosis. Therefore, treatment targeting hepatic fibrosis associated with schistosomiasis remains a challenging proposition.

The present study was designed to investigate the potential complementary schistosomicidal and hepatoprotective activities of the methanol extract of *Capparis spinosa* L. (*C. spinosa*) with or without praziquantel (PZQ) and compare results with silymarin (Milk thistle), a known hepatoprotective and antifibrotic agent, on induced liver fibrosis by experimental *Schistosoma mansoni* (*S. mansoni*) infection. Total polyphenols in the extract were determined using colorimetric assay.

C. spinosa L. caused a partial decrease in worm burden; a statistically significant reduction in hepatic and intestinal tissue egg load, what was associated histopathologically with decreasing in both the number and diameter of granulomas, as well as restoring serum aminotransferases (AST & ALT), alkaline phosphatase (ALP) and improving liver albumin synthesis. The best results were obtained in the group of mice treated with *C. spinosa* L. and PZQ together. Quantitative estimation of total polyphenols content using colorimetric assay showed that *C. spinosa* L. leaves contain higher concentration of polyphenolic compounds than fruits.

It was concluded that *C. spinosa* L. has a promising hepatoprotective and antifibrotic properties and could be introduced as a safe and effective therapeutic tool with PZQ in the treatment of schistosomal liver fibrosis. Nevertheless further studies on the mechanism of action of *C. spinosa* L. in chronic liver diseases may shed light on developing therapeutic methods in clinical practice.

Keywords: *Schistosoma mansoni*; *Capparis spinosa* L.; praziquantel; silymarin; hepatoprotective; colorimetric assay

Introduction

Schistosomiasis produced by *S. mansoni* continues to be an essential cause of parasitic morbidity and mortality worldwide and is the most common fibrotic disease due to inflammation and the deposition of scar tissue around parasitic eggs trapped in the liver (Burke *et al*., 2010). This disease is the second most important tropical disease after malaria and has significant impact on public health and socioeconomic problems in developing countries. (Abdel Aziz *et al*., 2011).

* – corresponding author

It is known that fibrosis may be reversible while cirrhosis is an irreversible pathological process, Therefore it is important to prevent the progression of fibrosis to cirrhosis. However, there is no effective anti-fibrotic drug to date and the efficacy of most treatments were not conclusive in clinical trials (Bataller & Brenner, 2005). Currently available treatment for many liver diseases is ineffective and is associated with many adverse effects, usually resulting in liver transplantation as the only choice of treatment. Novel compounds which prevent or halt scarring response are urgently needed to prevent the progression to end-stage liver disease (Albanis *et al*., 2003).

Praziquantel (PZQ), as a safe anti-schistosomal drug, has been used for more than 30 years (Fenwick *et al*., 2003). In general, specific treatment of schistosomiasis results in parasite elimination, and later on, a slight reduction in hepatic fibrosis that is attributed to parasite eradication (Homeida *et al*., 1991). However, loss of PZQ efficacy in schistosomiasis treatment was reported from Egypt, where the drug was being used aggressively (Ali, 2011).

Returning to nature was one of the suggested solutions for treatment of liver damage resulting from schistosomiasis. Referring to traditional medicine it was found that *C. spinosa* L. (Caper) a wild plant growing in Egyptian deserts was used by the Ancient Egyptians in the treatment of liver diseases (Tlili *et al*., 2011). Several claims about the usefulness of *C. spinosa* and its low toxicity were justified by experimental evidences. It was known by its anthelmintic activity in traditional Arab medicine and used to treat liver dysfunction. In Ayurvedeic medicine capers are recorded as hepatic stimulants and protectors, thus improving liver function (Sher & Alyemeni, 2010). Its extract is one of the constituents of Ayurvedeic preparation administered to treat preliminary cases of acute viral hepatitis and cirrhosis of liver and has shown encouraging results against viral infection. Bedouin heat leaves in butter and used this preparation against external parasitic disease of camel (Batanouny, 2005), for all these reasons *C. spinosa* L. was the plant of primary choice for this study.

C. spinosa L. is a perennial spiny bush that bears rounded, fleshy leaves and big white to pinkish-white flowers (Manikandaselvi & Brindha, 2014). Several biological activities and medicinal properties of *C. spinosa* L. were scientifically proved, it has anthelmintic, cytotoxic, anti-inflammatory (Al-Said *et al*.,1988; Zhou *et al.,* 2011), antiarthritic (Feng *et al*., 2011), antioxidant (Tesoriere *et al.,* 2007; Bouriche *et al*., 2011), antibacterial (Sherif *et al.,* 2013), antifungal (Lam *et al*., 2008), antidiabetic (Rahmani *et al*., 2014), antiviral, immunomodulatory (Arena *et al*., 2008), anticarcinogenic (Lam *et al*., 2008; Kulisic-Bilusic *et al*., 2012; Sherif *et al*., 2013), antihistaminic, chondroprotective, (Sher & Alyemeni, 2010), cardiovascular, hypolipidemic, antiallergic (Manikandaselvi *et al*., 2016) and antihepatotoxic activities (Gadgoli & Mishra, 1999; Satyanarayana *et al*., 2009; Bigoniya *et al*., 2013)

There is still intensive need for effective treatment of schistosomal liver fibrosis with minimal side effects. Thus, it was worthy to investigate the biological effect of *C. spinosa* L. which is one of the plants known in Ancient Egyptians Arab, Iranian, Unani, Chinese and Ayurvedic medicines. In the hope of finding a complementary natural product that has hepatoprotective and antifibrotic activity which support and benefit in the therapy of schistosomiasis. The present study, therefore, suggest full sustainable exploitation of *C. spinosa* with the aim to benefit Egypt from its flora and validate the use of this plant for the first time. Either alone or combined with the antischistosomal PZQ on schistosomal liver fibrosis and then comparing the results with silymarin, a known hepatoprotective and antifibrotic agent.

Material and Methods

Plant material

Leaves and fruits of *C. spinosa* L. were collected from Agiba in Marsa Matroh, Egypt, May 2016. Identification of the plants was kindly confirmed by Mrs. Threase Labib, senior botanist of plant taxonomy at El-Orman botanical garden, Cairo, Egypt. Voucher specimens No. (362014) has been deposited in the herbarium of Faculty of Pharmacy, Cairo University.

Preparation of methanol extract

A sample of *C. spinosa* L. leaves were separately air dried in the shade, powdered and kept in tightly closed amber colored glass container. 100 gm of dried leaves was extracted in methanol, then sonicated for 1 hour in an ultrasound bath, kept overnight and then filtered. The clear filtrate was freeze dried (El-Hawary *et al*., 2015).

Quantitative estimation of total polyphenols using colorimetric assay

Assay of total polyphenols was done using UV-visible spectrophotometer, Nicolet evolution 100 (Thermo) was used for recording UV spectra and measuring the absorbance in UV range.

Establishment of the calibration curve and sample preparation

A calibration curve was prepared using gallic acid as a standard at concentrations of 5, 10, 20, 30, 40, 50, 60, 70, 80, and 90 µg/ml in methanol.

Powdered air-dried samples of both leaves and fruit of *C. spinosa* L. were separately extracted, till exhaustion, with methanol. The solvent was evaporated to dryness. Stock solution (25 mg % in methanol) of leaves and fruits extracts were prepared.

The method adopted was based on measuring the intensity of the blue color developed when phenolic compounds make a complex with Folin-Ciocalteau reagent using gallic acid as a standard. The concentration of the total phenolics was calculated as gallic acid equivalent (GAE) with reference to a pre-established standard calibration curve. Folin-Ciocalteau reagent (100 µl) was added to a test tube containing 20 µl of the extract solution. The contents were mixed, and a saturated aqueous sodium carbonate solution (200 µl) was added. The volume was adjusted to 2 ml by the addition of 0.68 ml of milliQ water and the contents were mixed

Table 1. The absorbance of different concentrations of standard gallic acid.

Concentration (µg/ml)	Absorbance (760 nm)
5	0.037
10	0.069
20	0.146
30	0.24
40	0.3
50	0.39
60	0.45
70	0.533
80	0.58
90	0.701

vigorously and measured at 760 nm according to the method of Farag *et al*. (2014). For each concentration, three determinations were carried out and the average of the obtained absorbance was plotted versus the concentrations.

Experimental animals and infection

Laboratory-bred male, Swiss albino mice of CD-1 strain, each weighing between 18 – 20 grams were used in this study. Mice were maintained throughout the study in conditioned rooms at 21 °C and food with 24 % protein content. The animal experiment was carried out according to the internationally valid guidelines and the protocol of this study was approved by a scientific research ethics committee at the TBRI. The animal experiments were performed in accordance with the TBRI committee for laboratory animals' research guidelines [Schistosome Biological Supply Program (SBSP), Theodor Bilharz Research Institute (TBRI), Giza, Egypt]. Virulent Egyptian strain of *S. mansoni* cercariae were obtained from infected *Biomphalaria alexandria* snails, which were reared and maintained at SBSP, TBRI. Each mouse in infected groups was infected by subcutaneous injection with (60 ± 10) *S. mansoni* cercariae (Liang *et al*., 1987).

Drugs

Silymarin (Legalon®) purchased from (Chemical Industries Development (CID), Giza, Egypt under License of: Madaus GmbH. Germany) was given orally in a dose of 750 mg/kg/day (El-Lakkany *et al*., 2012) for 5 days/week for 8 weeks in the form of aqueous suspension in 2 % Cremophor El (Sigma Chemical Co., St. Louis, MO, USA). Praziquantel ® (Praziquantel-Sedico Pharmaceutical Co. 6th of October City, Egypt) was given orally in a total dose of 1000 mg/kg divided equally on two consecutive days (Gonnert & Andrews, 1977) during the 6th week post infection (PI), in the form of aqueous suspension in 2 % Cremophor El. *C. spinosa* L. methanol extract was given orally in a dose of 100 mg/kg/day (Satyanarayana *et al*., 2009) for 5 days/week for 8 weeks in the form of aqueous suspension in 2 % Cremophor El.

Experimental design

A batch of 60 male Swiss albino mice was infected and divided into 6 groups (I-VI) and one group was uninfected (VII; served as normal control) as follows:

Group I: Received vehicle (2 % Cremophor EL).
Group II: Received praziquantel.
Group III: Received *Capparis spinosa* L. extract.
Group IV: Received both *Capparis spinosa* L. extract and praziquantel.
Group V: Received silymarin.
Group VI: Received both silymarin and praziquantel.
Group VII: Uninfected mice were received vehicle (2 % Cremophor EL)

Table 2. Effect of *C. spinosa* L. and silymarine with/without praziquantel treatment on worm burden in mice infected with *S.mansoni.*

Groups	WORM BURDEN				
	Male worm	Female worm	Couple worm	Total worms	Reduction %
Infected (vehicle)	4.8 ± 0.56	2.60 ± 0.07	7 ± 0.58	21.40 ± 0.91	-
Inf + PZQ	0.67 ± 0.28*	0*	0.67 ± 0.28*	2 ± 0.28*	91
Inf + CAP	2.75 ± 0.5	1.75 ± 0.96	7 ± 0.82	18.5 ± 0.62	14
Inf+ PZQ + CAP	0.5 ± 0.12*	0*	0.25 ± 0.10*	0.75 ± 0.29*	96
Inf + SIL	3.50 ± 0.17	2 ± 0.41	6.50 ± 0.71	18 ± 0.84	16
Inf+ PZQ + SIL	0.67 ± 0.28*	0*	0.33 ± 0.20*	1.33 ± 0.12*	94

Data are presented as mean ± SEM (standard error of the mean) (n = 10 in each group).
*Significantly different from infected (vehicle) group at P < 0.001.

Table 3. Effect of *C. spinosa* L. and silymarine with/without praziquantel treatment on oogram pattern in mice infected with *S.mansoni.*

Groups	Oogram pattern (%)		
	Immature ova	Mature ova	Dead ova
Infected (vehicle)	48.60 ± 3.51	45.20 ± 3.27	6.20 ± 1.10
Inf + PZQ	2.67 ± 0.12*	2.33 ± 0.80*	95 ± 1.30*
Inf + CAP	39.25 ± 2.99	46.75 ± 2.99	14 ± 1.63
Inf+ PZQ+ CAP	0.75 ± 0.50*	1 ± 0.82*	98.25 ± 1.26*
Inf + SIL	50.5 ± 2.12	40 ± 2.54	9.5 ± 2.12
Inf+ PZQ + SIL	1 ± 0.33*	0.67 ± 0.18*	98.33 ± 1.53*

Data are presented as mean ± SEM (n = 10 in each group)
* Significantly different from infected (vehicle) group at P < 0.001

All mice were sacrificed 8 weeks from day zero experiment by decapitation. After decapitation, blood samples collected in centrifuge tubes were centrifuged at 3000 rpm for 20 min. Serum was stored at -20 °C until used for biochemical assays. Porto-mesenteric perfusion was performed for recovery of *S. mansoni* worms in infected mice for subsequent counting (Duvall & De Witte, 1967). Pieces of intestine and liver were digested in 5 % KOH (potassium hydroxide) solution and number of eggs per gram intestine or liver was calculated to determine the ova count in tissue (Cheever, 1968). The percentage of different egg developmental stages (Oogram pattern) were studied according to the method of Pellegrino *et al*. (1962) in which eggs at different stages of maturity were identified and counted.

Assessment of toxicity and determination of LD_{50}
A group of 18 adult (6 weeks old) normal male CD-1 Swiss albino mice weighing between 20 – 25 g were used to study the toxicity effect and to determine the LD_{50} of *C. spinosa* extract. They were subdivided into six subgroups of three mice each. Subgroups were treated orally with increasing doses of 100, 500, 1000, 2000, 3000 and 4000 mg/kg plant extract re-suspended in 2 % Cremophore EL (Sigma/Aldrich Chemical Co., St. Louis MO, USA). Mortality rates were recorded 24 hours post treatment. The death rate was used to judge toxicity.

Assessment of biochemical parameters
The Biodiagnostic kits at Dokki laboratory (Giza, Egypt) were used for the determination of serum aminotransferase enzymes (AST and ALT) activities, ALP and serum albumin.

Histopathology and Granuloma Measurement
Livers recovered from mice were fixed in 10 % buffered formalin

Table 4. Effect of *C. spinosa* L. and silymarine with/without praziquantel treatment on tissue egg load in mice infected with *S.mansoni.*

Groups	Tissue egg load/gm × 10^3			
	Hepatic tissue		Intestinal tissue	
	Egg load	Reduction %	Egg load	Reduction %
Infected (vehicle)	29.69 ± 0.31	-	31.61 ± 0.72	-
Inf + PZQ	5.79 ± 0.83*	80	0.98 ± 0.36*	97
Inf + CAP	16.26 ± 0.86*	45	12.17 ± 0.95*	62
Inf + PZQ + CAP	1.06 ± 0.36*	96	0*	100
Inf + SIL	19.62 ± 0.53*	34	8.63 ± 0.30*	30
Inf + PZQ + SIL	0.50 ± 0.38 *	98	0*	100

Data are presented as mean ± SEM (n = 10 in each group).
* Significantly different from infected (vehicle) group at P < 0.001.

and processed to paraffin blocks. Sections (4 µm thick) were cut 250 µm away from the preceding sections to avoid measurement of the same granuloma. Five paraffin liver sections were prepared from each animal and stained with haematoxylin and eosin (H&E) and Masson trichrome stains. Measurements of the granuloma size were conducted on non-contiguous granulomas, each containing a single egg (with intact or degenerated miracidia), using an ocular micrometer. The mean diameter of each granuloma was calculated by measuring two diameters of the lesion at right angles to each other (Von Lichtenberg, 1962). For each mouse, 40 granulomas were measured and associated hepatic histopathological changes were studied.

Statistical Analysis of Data

Data were reported as mean counts ± standard deviation. Statistical analyses were done using computerized statistical software program IBM SPSS 19.0. The independent sample t-test was used to assess the statistical significance of the difference between two study group means. Statistical significance was defined as *P* values < 0.05.

Results

Results of quantitative estimation of polyphenols content using colorimetric assay

The absorbance of different concentrations of standard gallic acid was illustrated in Table 1. The present study revealed that *C.spinosa* L. leaves contain higher concentration of phenolic compounds (28.4 µg/ml) than fruits (7.3 µg/ml).

Parasitological results

Praziquantel resulted in the highest percentage of worm burden reduction (91 %) when administered alone to mice infected with *S. mansoni.* This percentage increased to (96 %) and (94 %) after the synergistic effect of silymarin and *C. spinosa* extract, respectively. Infected animal groups treated with praziquantel alone, praziquantel combined with *C. spinosa* extract and praziquantel combined with silymarin extract showed significantly different effects on worm burden when compared with infected group (i.e male worm, female worm, coupled worm and total worms, at $P < 0.001$). Generally female worms were more affected by drugs used than male worms. They died when treated with praziquantel alone, or combined with *C. spinosa* extract and silymarin extract. Meanwhile the administration of *C. spinosa* extract alone or silymarin extract alone to the *S. mansoni* infected animals yielded in the lowest worm burden reduction, 14 % and 16 % respectively. The effect of *C. spinosa* L. and silymarin with/without praziquantel on the worm burden of mice infected with *S.mansoni* is shown in Table 2.

All egg stages (immature, mature and dead) were observed in the intestines of infected non-treated and treated mice. As presented in Table 3, theywere evidently affected by administration of praziquantel alone and showed a significant reduction in the percentage of dead ova (95 ± 1.30 %) when compared with groups administered with *C. spinosa* extract alone (14 ± 1.63 %) or silymarin extract alone (9.5 ± 2.12 %).

Administration of praziquantel alone to mice infected with *S. mansoni* showed the highest percentage in reduction of ova (80, 97 %), what was followed by *C. spinosa* extract alone (45, 62 %) and silymarin extract alone (34, 30 %) in both liver and intestinal tissues, respectively. The reduction percentage of ova count increased to 100 % in intestinal tissues after administration of praziquantel combined with *C. spinosa* extract or praziquantel combined with silymarin extract. Also, this reduction percentage in ova count was observed in hepatic tissues when treated with both praziquantel and *C. spinosa* extract (96 %), or praziquantel and silymarin extract (98 %). These effects of *C. spinosa* L. and silymarin with/ without praziquantel on tissue egg load in mice infected with *S. mansoni* are presented in Table 4.

Acute Toxicity and Determination of LD_{50}

Zero mortality wase observed 24 hours, as wellas post treatment with increasing doses of C. spinosa extract starting from 100 mg/ kg to 4000 mg/kg, LD_{50} >4000 mg/ kg. That means that *C. spinosa* L. extract is safe at the treatment dose of 100 mg/ kg.

Biochemical Parameters Assessments

Infection of mice with *S. mansoni* resulted in defective excretion and elevation of serum AST, ALT and ALP values due to livers damag. Also a serious decrease in serum alumin was observed. These results were significantly different from uninfected (vehicle) group . Administration of praziquantel, *C. spinosa* extract and silymarin extract either alone or in combination with praziquantel to the infected animals with *S. mansoni* showed an improvement in all liver functions which were significantly different when compared with infected (vehicle) group ($P < 0.05$). Except liver albumin the Infected animal group treated with praziquantel alone showed slightly better improvement in all tested liver functions when compared with other groups treated with *C. spinosa* extract alone or silymarin extract alone, The best results of serum albumin level were obtained when praziquantel combined with *C. spinosa* extract (3.36 ± 0.04 gm/dl). This result was nearly the same of that of the uninfected animal group (3.47 ± 0.15 gm/dl), Table 5 summarizes all these results.

Histopathological Study

Stained liver sections with haematoxylin and eosin (H&E) and Masson's trichrome (MT) of *S. mansoni* infected not treated mice demonstrated that liver tissue was studded with granulomas, many granulomas were fused together with intact eggs, also ballooned hepatocytes were diffusely seen. Hepatic tissues of the infected mice treated with PZQ display moderate degree of improvement with presence of many granulomas, loose fibrous reaction, less inflammatory aggregates and vasculature showed congestion together with the focal necrotic area. Hepatic tissues of infected mice treated with *C. spinosa* L. display mild to moderate degree of improvement, most granulomas appeared with dead egg, less fi-

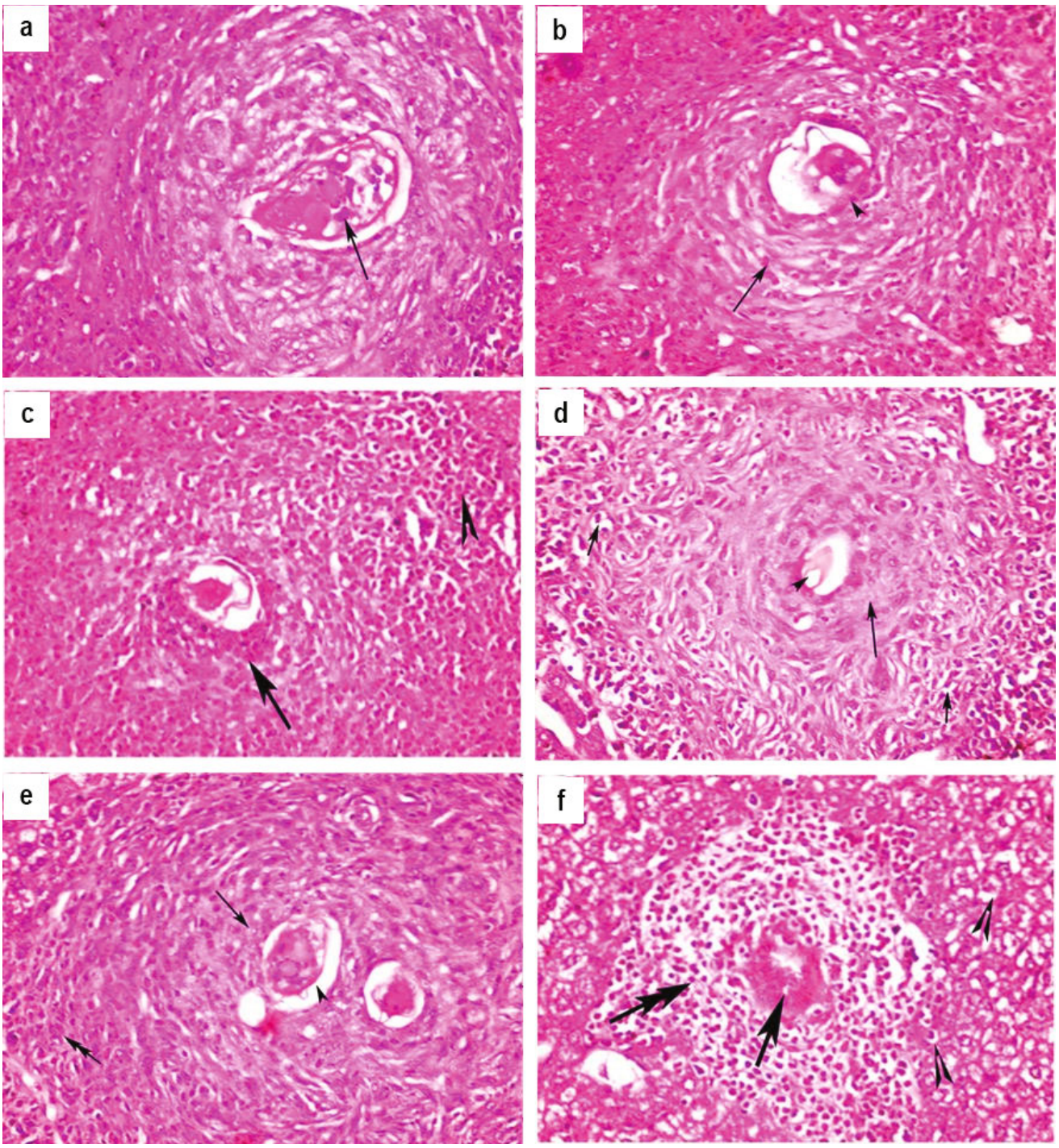

Fig. 1. Histopathological examination of haematoxylin and eosin stained liver sections (a) Infected control group demonstrating large fibrocellular granuloma surrounding one intact egg (arrow) and peripheral zone of inflammatory cells, (b) Infected mice treated with PZQ demonstrating granuloma with loose fibrotic reaction (arrow) and dead egg (arrow head), (c) Infected mice treated with *C. spinosa* L. extract demonstrating notable reduction in granuloma size, with loosening of the central fibrous area of granulomatous reaction (arrow) and dense inflammatory cellular response (arrow head), (d) Infected mice treated with *C.spinosa* L. extract and PZQ demonstrating moderate loose of fibrotic reaction (arrow), dead egg (arrow head) and less cellular inflammatory infiltrate around (short arrow), (e) Infected mice treated with silymarin demonstrating compact granuloma with dense fibrotic reaction (arrow), intact egg (arrow head) and inflammatory area (double arrow), (f) Infected mice treated with silymarin and PZQ focusing on granuloma with dead egg (arrow), loose inflammatory infiltration (double arrow), hepatocyte with vacuolated cytoplasm (arrow head) (x: 400).

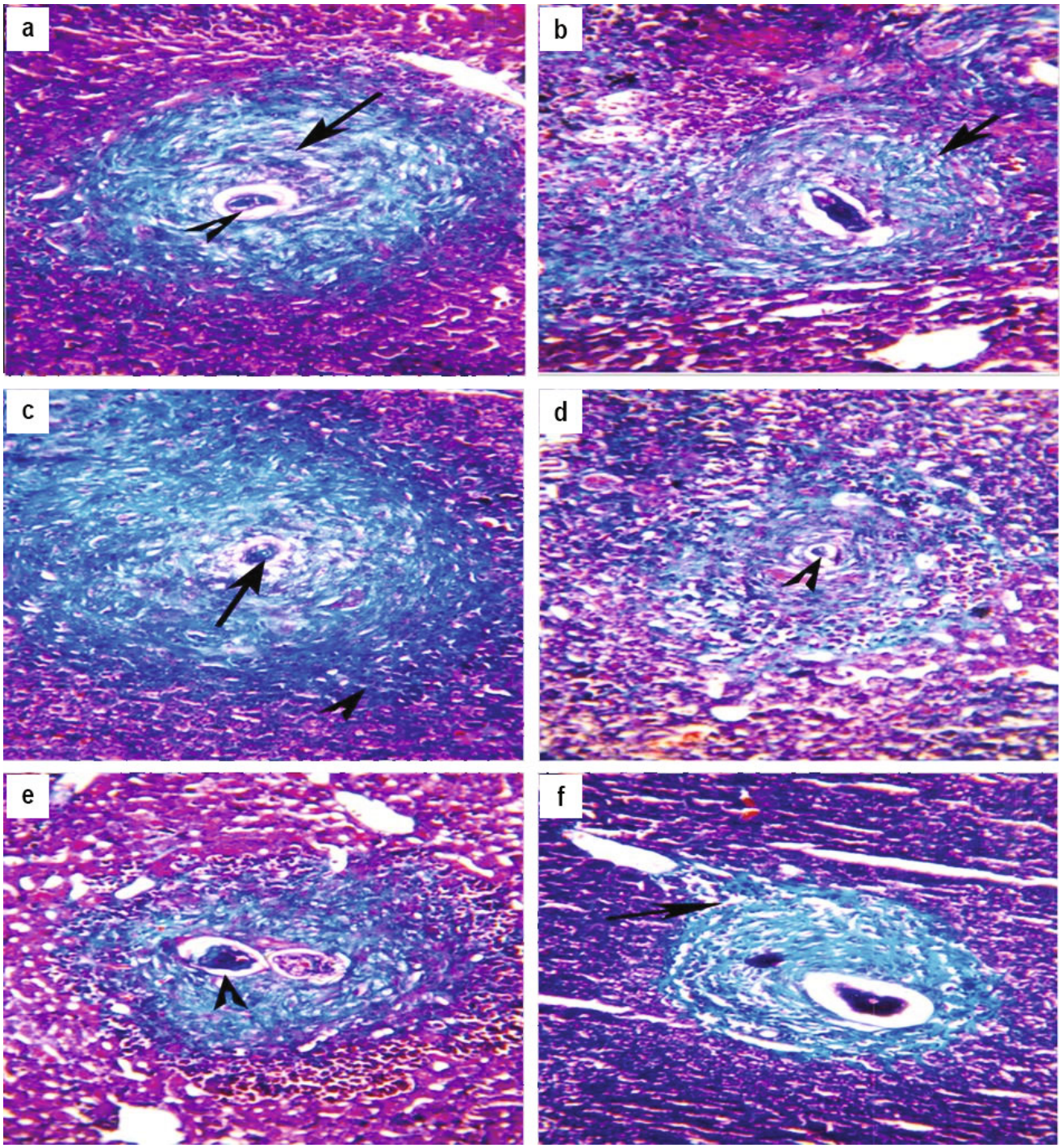

Fig. 2. Histopathological examination of liver sections from (a) Infected control group demonstrating granuloma with compact fibrous reaction (arrow) intact egg (arrow head) and moderate inflammation, (b) Infected mice treated with PZQ demonstrating loose fibrous area and mild degree of inflammatory reaction (arrow), (c) Infected mice treated with *C. spinosa* L. extract demonstrating granuloma with loose fibrosis, dead egg (arrow) and external layer of inflammatory cells (arrow head), (d) Infected mice treated with *C.spinosa* L. extract and PZQ demonstrating granuloma with loose fibrotic reaction and dead egg (arrow), (e) Infected mice treated with silymarin demonstrating granuloma with intact egg (arrow head) and compact surrounding granuloma, (f) Infected mice treated with silymarin and PZQ demonstrating loose of fibrous area around dead egg and less inflammatory reaction (arrow) [Masson's Trichrome x: 400].

Table 5. Effect of *C. spinosa* L. and silymarine with/without praziquantel treatment on liver function tests 8 weeks post *S.mansoni* mice infection.

Animal Groups	Serum AST (U/L)	Serum ALT (U/L)	Serum Albumin (gm/dl)	Serum ALP (U/L)
Uninfected (vehicle)	42.67 ± 0.52	26.33 ± 1.53	3.47 ± 0.15	32.33 ± 1.15
Infected (vehicle)	111.8 ± 2.86 *	53.8 ± 0.38 *	1.9 ± 0.27 *	87 ± 1.41 *
Inf + PZQ	70.83 ± 0.64 **	39.4 ± 1.09 **	2.94 ± 0.11 **	59 ± 1.43 **
Inf + CAP	81.5 ± 1.11 **	45.5 ± 1.29 **	3.03 ± 0.51 **	65.75 ± 1.06 **
Inf + PZQ + CAP	62.25 ± 1.59 **	30.75 ± 0.84**	3.36 ± 0.04 **	48.75 ± 1.71 **
Inf + SIL	89.5 ± 0.71 **	49.75 ± 0.35 **	2.75 ± 0.07 **	73 ± 1.14 **
Inf + PZQ + SIL	67.17 ± 0.76 **	35.5 ± 0.5 **	3.1 ± 0.5 **	54.67 ± 1.08 **

Data are presented as mean ± SEM (n = 10 in each group).
ALT, Alanine aminotransferase; AST, Aspartate transaminase; ALP, Alkaline phosphatase.
*Significantly different from uninfected (vehicle) group at P < 0.05. ** Significantly different from infected (vehicle) group at P < 0.05.

brosis, peri-inflammatory layers, and hepatocytes revealed moderate cytoplasmic vacuolation with vesicular nuclei or marginated chromatin. Hepatic tissues of infected mice treated with *C. spinosa* L. and PZQ revealed mild to moderate ameliorating impact, where many granuloma seen with loose reaction and dead eggs, a notable reduction in the number of granuloma and patchy areas of hepatic cells showed vacuolated cytoplasm. Silymarin alone has no effect on schistosoma granuloma, but the most effective impact was on hepatocytes where wide areas of intact hepatocytes were detected. However, ballooned hepatocytes in pericentral areas could be observed together with an increase in the extracellular matrix. Hepatic tissues of infected treated mice with PZQ and silymarin showed a notable reduction in the number of granulomas and hepatic cells showed loose mild vacuolated cytoplasm as shown on Figs. 1 and 2. The number and diameter of hepatic granulomas in different experimental groups are shown on Table 6.

Discussion

Medicinal plants are used worldwide for centuries to promote and maintain health as well as to relief symptoms in chronic diseases. Polyphenols are phytochemical compounds found in plants which apparently have vital health benefits and prevent from health dangers such as cancer, high blood pressure, heart disease or diabetes (Rose, 2013). In general they are potent antioxidants, metal chelators with anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Tapas *et al*., 2008).

In the present study, a quantitative estimation of total polyphenolic content using colorimetric assay revealed that *C. spinosa* L. leaves contain higher concentration of polyphenols than fruits, so the methanol extract of the leaves to estimate its biological activity was used.

Table 6. Effect of *C. spinosa*. L. and silymarine with/without praziquantel treatment on hepatic granuloma number and diameter 8 weeks post *S.mansoni* mice infection.

Groups	Mean number of granuloma in 10 successive power fields	Mean granuloma diameter in µm
Infected (vehicle)	11.63 ± 1.52	347.34+17.24
Inf + PZQ	5.26 ± 1.26*	231.56 ± 23.34*
Inf + CAP	7.33 ± 2.12*	239.34 ± 14.45*
Inf + PZQ + CAP	4.29 ± 1.34*	212.74 ± 24.54*
Inf + SIL	6.14 ± 1.14*	234 ± 16.62*
Inf + PZQ + SIL	3.25 ± 0.12*	207 ± 13.34*

Data are presented as mean ± SEM (n = 10 in each group).
* Significantly different from uninfected (vehicle) group at P < 0.05.

These results matched with the studies carried out by Yu *et al*. (2006) who isolated some phenolic compounds from the fruit of *C. spinosa* L. by chromatographic methods. Also Kulisic-Bilusic *et al*. (2012) evaluated the aqueous infusion of caper and showed an interesting compositional pattern containing several phenolic compounds reflecting the potential benefits of the plant.

In this study, the evaluation of parasitological parameters was done to confirm that all mice had received unbiased *S. mansoni* infection. In addition the presence of hepatic granulomas around trapped *schistosome* eggs affecting hepatocellular functions was examined to confirm the eradication of the worms and eggs by PZQ. Histopathological examinations of hepatic sections of sacrificed mice in all groups, by staining with haematoxylin and eosin and Masson's trichrome stains, for detection of the histopathological changes and fibrosis degree were done to assess the cure rates and degree of liver tissue healing after the treatment.

In this research, treatment of *S. mansoni* infected mice with PZQ resulted in a 91 % reduction in worm burden. This was accompanied with significant reduction in the hepatic and intestinal tissue egg load by 80 % and 70 %, respectively. There was also a significant increase in the percentage of dead eggs when compared with the infected untreated group. These results are consistent with several previous studies accomplished by Issa (2007), Abdel-Rahman (2009), El-Sisi *et al*. (2011) and El-Lakkany *et al*. (2012). The main justification for these results is that PZQ affects adult schistosomes directly. This leads to their contraction, paralysis and subsequent shift from mesenteric to hepatic veins where they are finally damaged by the phagocytic cells. In addition, PZQ causes destruction of the worm tegument leading to the exposure of tegumental antigen, which becomes easily accessible to the parasites' specific antibody (Fallon *et al*., 1995). Administration of PZQ showed a significant improvement in all hepatic functions and associated histopathology with a significant decrease in the number and diameter of granulomas. These findings are in agreement with El-Sisi *et al*. (2011) and El-Lakkany *et al*. (2012).

Remarkably, treatment of *S. mansoni* infected mice with *C. spinosa* L. alone resulted in reduction in worm burden by 14 %, which was accompanied with a statistically significant reduction in hepatic tissue egg load by 45 % and a statistically significant reduction in intestinal tissue egg load by 62 % when compared to the infected untreated group.

In addition, administration of *C. spinosa* L. resulted in some healing of hepatic granulomatous lesions what was supported parasitologically by partial increase in the percentage of dead ova and associated histopathology by decrease in diameter of granulomas, more ova degeneration and fewer inflammatory cells. This may be attributed to the anthelmintic, anti-inflammatory, antioxidant, antihistaminic, immunomodulatory, and antihepatotoxic activities of *C. spinosa* (Manikandaselvi *et al*., 2016). Thus, it is possible that *C. spinosa* L. eliminates the products of oxidative reactions and assists in the immune-mediated destruction of worms and eggs.

PZQ is an active anti-schistosomal drug, against all forms of schistosomiasis (Ali, 2011). To stimulate further PZQ healing outcome, which could diminish liver fibrosis simultaneously with worm removal. it was recommended to develop a combination of PZQ and an antifibrotic drug in the treatment of murine schistosomiasis (Doenhoff *et al*., 2002). Co-administration of *C. spinosa* L. with PZQ also showed a statistically significant decrease in worm burden (96 %) and no viable eggs were present, associated with reduction in hepatic tissue egg load by 96 % and 100 %, respectivealy. The reduction of intestinal tissue egg load and better healing of hepatic granulomatous lesions indicates that the use of *C. spinosa* L. along with PZQ did not interfere or affect the antischistosomal effect of PZQ.

The administration of *C. spinosa* L. either alone or in combination with praziquantel showed a statistically significant reduction in the elevated liver enzymes ALT, AST and ALP. This reduction in serum transaminases (AST and ALT) and ALP values reveals that *C. spinosa* L. has a hepatoprotective activity. These protective effects might be as a result of plasma membrane stabilization and preservation the structural integrity of cells as well as repair of hepatic tissue damage caused by *S. mansoni* infection. Also, there was a significant elevation of the level of serum albumin.

Determination of enzyme levels, like serum AST and ALT is essential for the evaluation of liver damage by schistosomal infection (Aly & Mantawy, 2013 and Al-Sayed *et al*., 2014). Necrosis or membrane damage releases the enzymes into circulation; so, they can be measured in the serum. In agreement with the reports of Kadry *et al*. (2013) and Mahmoud & Elbessoumy (2013), the raise of these enzymes in serum may be due to the hepatocytes damage caused by the parasite eggs toxins released into the circulation. In addition, Naik *et al*. (2011) reported that following schistosomal infection the hepatocyte membrane damage seems to be the prime culprit for the marked increase of the serum enzymes such as AST, ALT, and ALP. Also, hypo-albuminemia may be due to malabsorption resulting from damage of intestinal mucosa due to the extrusion of great numbers of eggs or could be due to diminished synthesis, which may result from parasitic injury to hepatic cells (Oliveira *et al*., 2009).

This study concurred with the study performed by Satyanarayana *et al.* (2009) who studied the hepatoprotective effect of the alcohol extract of *Capparis sepiaria* Linn. Capparaceae stem against carbon tetrachloride (CCl_4) -induced toxicity in albino rats. The rats were administrated daily pretreatment with an alcohol extract of *C. sepiaria* (100 mg/kg) and the standard silymarin (25 mg/kg) orally for 7 days. The toxicant used on 7th day was CCl4 at a dose of 1.25 ml/kg as 1:1 mixture with olive oil. The extract resulted in a significant ($p<0.01$) reduction of the elevated levels of aspartate transaminases (AST and ALT) total bilirubin and rise of the decreased total protein level when compared with the toxic control.

El-Lakkany *et al*., (2012) studied the effect of silymarin on *S. mansoni* induced liver fibrosis. It was reported that silymarin has a favorable anti-inflammatory and anti-fibrotic activity. Its application caused a partial decrease in worm burden; hepatic tissue egg

load, reduced the elevated ALT, and when it was combined with PZQ treatment, a complete eradication of worms, eggs and relieved liver inflammation and fibrosis were observed.

Regarding silymarin treatment, it was noticed that there was no significant difference between treatments with *C. spinosa* L. extract or silymarin alone or in combination with praziquantel. Silymarin promotes liver health through its anti-oxidant, anti-inflammatory, anti-proliferative, and immuno- modulatory effects (Polyak *et al*., 2013).

The choice of PZQ and silymarin as suitable drugs used for the treatment of schistosomiasis and liver fibrosis was matched with many previous studies. Rabia *et al*. (2010) proved that treatment with PZQ alone or combined with silymarin resulted in significant reduction of parasitological parameters whereredуction in worm burden was associated with a reduction in tissue egg load and changes in the oogram pattern. These results were confirmed also by our study.

Numerous previous studies confirmed that phenolic compounds isolated from *C. spinosa* L. possess hepatoprotective activity. For example Gadgoli and Mishra (1999) isolated *p*-methoxybenzoic acid (Phenolic compound) from *C. spinosa* L. leaves. It possessed significant antihepatotoxic activity against carbontetrachloride and paracetamol induced hepatotoxicity *in vivo* and thioacetamide and galactosamine induced hepatotoxicity in isolated rat hepatocytes. Bigoniya *et al.* (2013) found that kaempferol (flavonoid) isolated form *C. spinosa* L. has a potent hepatoprotective action upon CCl_4 induced oxidative stress and liver toxicity in rat. The hepatoprotective effect of kaempferol can be correlated with its efficiency to normalize the levels of serum marker enzymes and enhance the antioxidant defiance status. The findings suggest that kaempferol can be used as a safe and effective alternative chemopreventive agent in the management of liver disorders.

In conclusion, *C. spinosa* L. leaves extract has a promising hepatoprotective and antifibrotic properties and this effect may be attributed to the relatively high contents of polyphenols. It could be introduced as a safe and effective therapeutic tool with PZQ in the treatment of schistosomal liver fibrosis. Further studies are clearly warranted to investigate the efficacy of higher doses and longer duration of treatment of *C. spinosa* L. leaves extract in this model of liver fibrosis, and thus establish their clinical applicability in patients with chronic liver diseases.

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Prevalence of parasitic infections in surgically removed appendices: parasitological and histopathological studies

A. S. AMER[1], A. E. SAAD[1]*, S. N. ANTONIOS[1], E. A. HASBY[2]

[1]Department of Medical Parasitology, Faculty of Medicine, Tanta University, E-mail: *abeerezzat62@gmail.com*;
[2]Department of Pathology, Faculty of Medicine, Tanta University

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Summary

Intestinal parasites may cause symptoms similar to acute appendicitis. Moreover, the diagnosis of parasitic infections is only done by post-operative histopathological examination of the appendices. Therefore, our aims are to assess the prevalence of intestinal parasitic infections among patients who were be appendectomized at Tanta Hospitals, Egypt and to investigate the possible association between these parasitic infections and appendicitis. To achieve these objectives, we performed a cross-sectional study including 65 patients chosen randomly who had undergone appendectomy over a period of one year from Oct 2015 to Oct 2016. Demographic data were retrieved. Complete blood picture was done. Moreover, appendiceal faecolith were examined macroscopically then by direct smear examination, formol-ether concentration technique, modified Ziehl-Nelseen stain and rapid immunochromatographic test. Histopathological examination of resected appendices was done. We found that parasitic infections were detected in 24.6 % of examined cases. Most of parasitic infections were prevalent in patients belonging to the school age group. Different parasitic infections were detected in the faecolith specimens. Moreover, *Enterobious vermicularis* adult female and *Schistosoma mansoni* granuloma were detected in histopathological sections. Also, a spectrum of pathological changes in the appendices was found ranging from lymphoid hyperplasia to acute inflammation with peritonitis. In conclusion, intestinal parasites may cause clinical picture similar to that of acute appendicitis. Therefore, careful attention to clinical history, stool examination and high eosinophilia may aid diagnosis and avoid unnecessary appendectomy. Moreover, the presence of different parasitic stages in the narrow lumen of the appendix may have a role in the development of appendicitis and this needs further studies.

Keywords: appendicitis; immunochromatographic test; intestinal parasites; *Hymenolepis nana*; *Enterobious vermicularis*; appendiceal faecolith

Introduction

Parasitic infections are common on a worldwide basis and are seen in high numbers of developing countries especially in Africa where a warm, moist climate and standards of hygiene are low. However, parasitic diseases are now occurring in developed countries in higher frequency than before due to immigration and increased foreign travel (Jan *et al*., 2010).

Many health problems result from these infections, including malnutrition, iron-deficiency anemia and surgical morbidities such as intestinal obstruction, cholecystitis, liver abscess and appendicitis which need surgical intervention (Hesse *et al*., 2012).

* – corresponding author

Table 1. Distribution of parasites in relation to the age and gender of positive studied cases.

Age group	N.	%	P value	Gender		Positive	X^2	P-value
6 – 18 y.	10	62.5	0.157	Male	N	9	12.802	0.001*
					%	90 %		
				Female	N	1		
					%	10 %		
> 18 y.	6	37.5		Male	N	4	1.332	0.248
					%	66.67 %		
				Female	N	2		
					%	33.33 %		

* extremely significant

Acute appendicitis is a common cause of an emergent surgical intervention. The prevalence of appendicitis is reported to be around 7 %, being slightly higher in male patients. It can be seen at any age but the peak incidence occurs in older children and young adults (Yabanoglu *et al*., 2014).

In the 21st century the incidence of appendicitis is higher in newly industrialized countries in Asia, South America and the Middle East as compared with Western countries due to different environmental exposures (Ferris *et al*., 2017).

There are many theories for pathogenesis of appendicitis; one of these theories is the obstruction of the lumen of the appendix. The other theory is really a group of theories, which assumes the existence of a labile factor in the appendix that responds to a variety of external and internal stimuli that causes injury to the appendix which permits bacterial invasion by the flora normally present (Bohrod,1946). Moreover, changes in symbiont composition in the ecosystem of the human body have led to immune dysfunction and subsequent disease (The biota alteration theory, or biome depletion theory); that may predispose to appendicitis (Parker, 2017).

These theories may account for the association of appendicitis with distant infections and the influence of climatic and other environmental alterations.

It has been reported that the cause of appendicitis is intraluminal obstruction caused by lymphoid hyperplasia, parasite-associated fecal matter and ingested foreign bodies (Pasupati *et al*., 2008).

Some parasites such as *Enterobius vermicularis, Ascaris lumbricoides*, *Schistosoma* spp. and *Taenia* spp. are reported in the appendectomy specimens. Also, some protozoa are reported such as *Entamoeba histolytica*, *Balantidium coli* and *Cryptosporidium parvum* (Hegazi, 2012).

Therefore, the current study aimed to assess the prevalence of intestinal parasitic infections among patients who were appendectomized at Tanta University Hospitals, Egypt and to investigate the possible association between these parasitic infections and appendicitis through parasitological and histopathological examinations of the surgically removed appendices.

Patients and Methods

A cross-sectional study was conducted within one year from October 2015 to October 2016 on 65 cases of appendictomized patients chosen randomly after taking their consent. Arrangement with General Surgery department, Tanta University Hospitals to obtain the appendices after the surgery was done. Interviewing questionnaire including demographic data such as age, sex,

Table 2. Detected parasites in positive studied cases.

Parasites in positive cases	Methods of detection	No.	%
Enterobius vermicularis	Histopathological examination of appendiceal specimens	1	6.25 %
Schistosoma mansoni		1	6.25 %
Hymenolepis diminuta	Microscopic examination of appendiceal faecolith	1	6.25 %
Hymenolepis nana		2	12.5 %
Giardia duodenalis		4	25 %
Entamoeba histolytica		4	25 %
Cryptosporidium spp.		2	12.5 %
Blastocystis spp.		1	6.25 %
Total		16	100 %

name, level of education, residence and clinical data such as abdominal colic, vomiting and diarrhea was done.

Parasitological study

The luminal contents of surgically removed appendices (appendiceal faecolith) were examined macroscopically for consistency, color, odor and the presence of blood, mucus and parasites. Also, they were examined microscopically through:

Direct smear examination: One or two drops of saline was added to the faecolith with a pipette and mixed with pipette tip. The specimen was examined with the low power objective lens (10x) and low light according to Fleck and Moody (1993).

Preserved in 10 % formalin and examined later by formal-ether concentration technique to diagnose intestinal parasitic ova and cysts according to Fleck and Moody (1993).

Stained by modified Ziehl-Nelseen stain to detect intestinal protozoa. Faecal smears were stained with strong carbol fuchsin for 15 – 20 minutes. They were decolorized in acid alcohol (1 % HCl in methanol) for 15 – 20 seconds. Then, they were counterstained with 0.4 % malachite green (or methylene blue) for 30 – 60 seconds according to Rosenblatt *et al*. (2009).

Copro antigen of C*ryptosporidium* spp., *Giardia lamblia* and *Entamoeba histolytica* were detected in these appendiceal faecolith by rapid immunochromatographic test (Rida Quick *Cyptosporidium/ Giardia/ Entamoeba* Combi Art No, N1723) according to Regnath *et al*. (2006). The test was performed according to manufacturer's instructions. Results were interpreted following the manufacturer's guidelines.

Any appendiceal specimens or faecolith were discarded properly after testing by safe disposal measures in Tanta Faculty of Medicine general incinerator according to the safe disposal rules.

Laboratory blood picture

Blood samples were collected from patients and complete blood picture was done to detect anemia, leukocytosis and eosinophilia. The blood film was fixed with methyl alcohol for 2 minutes. Giemsa

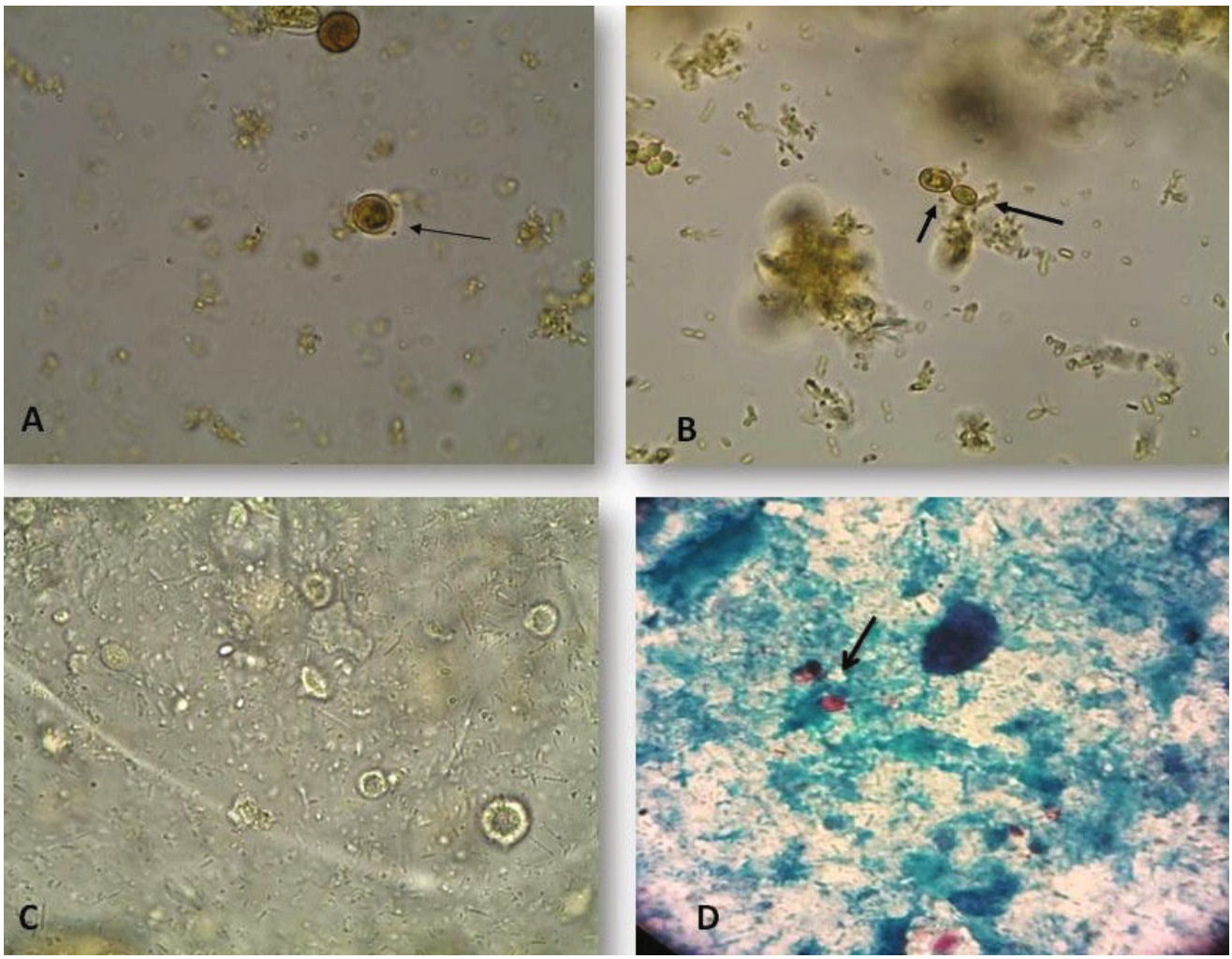

Fig. 1. Luminal content of appendiceal specimens showing: A: *Entamoeba histolytica* cyst with concentrated iodine stain (x1000). B: *Giardia duodenalis* cyst with concentrated iodine stain (x1000). C: *Blastocystis* cyst with concentrated iodine stain (x400). D: *Cryptosporidium* oocysts with modified Ziehl-Nelseen stain (x1000).

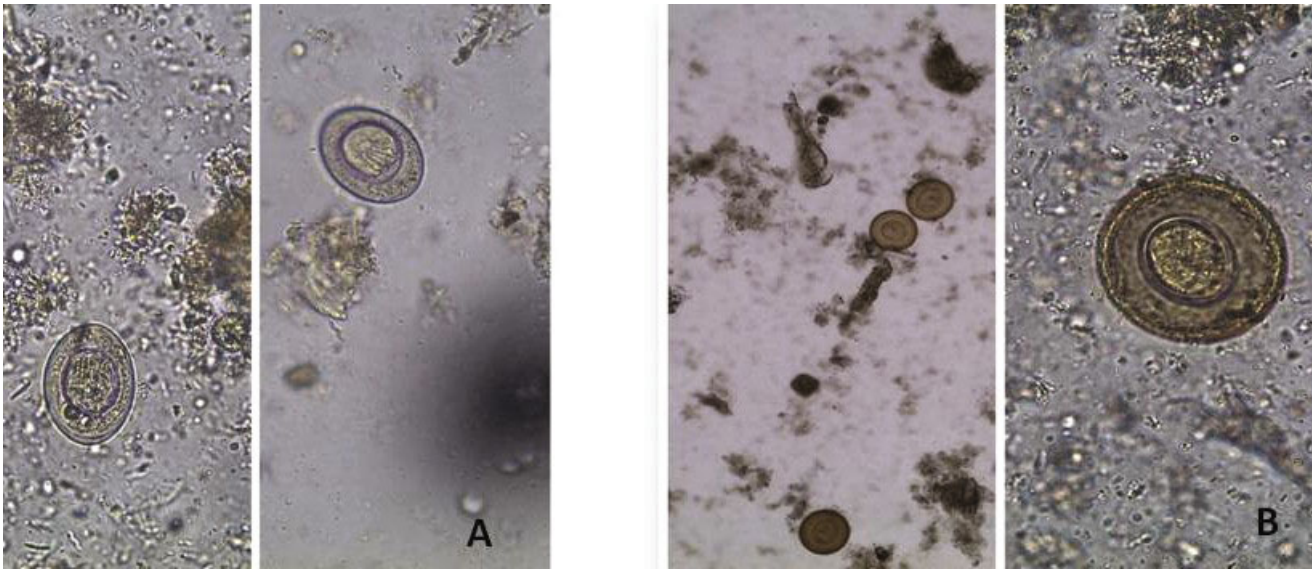

Fig. 2. Luminal content of appendiceal specimens with concentrated iodine stain showing: A: *Hymenolepis nana* ova (x400). B: *Hymenolepis diminuta* ova (x100) (left) and (x400) (right).

stain 1:9 dilution was poured with buffer over the smear for 8 – 10 minutes. Then, the film was washed off with buffer and dried. The dry and stained films were examined without a coverslip under oil immersion objective (Houwen, 2002).

Histopathological examination

Two transverse sections from the base and the middle portion of the appendix and a longitudinal section from the tip of the appendix were taken. Sections of 4 µm thickness were prepared and stained with haematoxylin and eosin. The histologic diagnosis was confirmed by reviewing one to four original sections of the specimen. Stained sections of appendiceal specimens were subjected to microscopic examinations to detect any pathological changes such as inflammation, granuloma of the appendix and the presence of parasites (Allen, 1992).

Statistical analysis

Quantitative values of the measured parameters were expressed as mean ± standard deviation (SD). The data were analyzed by one way-ANOVA to determine significance of differences between groups using Statistical Package for Social Sciences (SPSS), version 14.0. The probability of significant differences was determined by chi-square test for the histopathological studies. Differences were considered significant at $P < 0.05$ and extremely significant at $P < 0.001$.

Results

Parasitic infections, helminth and protozoa, were found in 16 (24.6 %) of cases in this study either by feacolith or tissue sections examined. The present study was conducted on two age groups: school age (6 – 18 years, 60 % of appendectomized cases) and

Table 3. Sensitivity and specificity of different diagnostic techniques for stool examination.

	Giardia duodenalis		*Entamoeba histolytic*a		*Cryptosporidium* spp.	
	Formol ether technique	Immunodiagnostic technique (Copro antigens)	Formol ether technique	Immunodiagnostic technique (Copro antigens)	Formol ether technique	Immunodiagnostic technique (Copro antigens)
Sensitivity	75	100	100	100	-	-
Specificity	-	-	100	-	100	100
PPV	100	100	100	75	-	-
NPV	-	-	100	-	100	100
Accuracy	75	100	100	100	100	100

PPV: positive predictive value
NPV: Negative predictive value

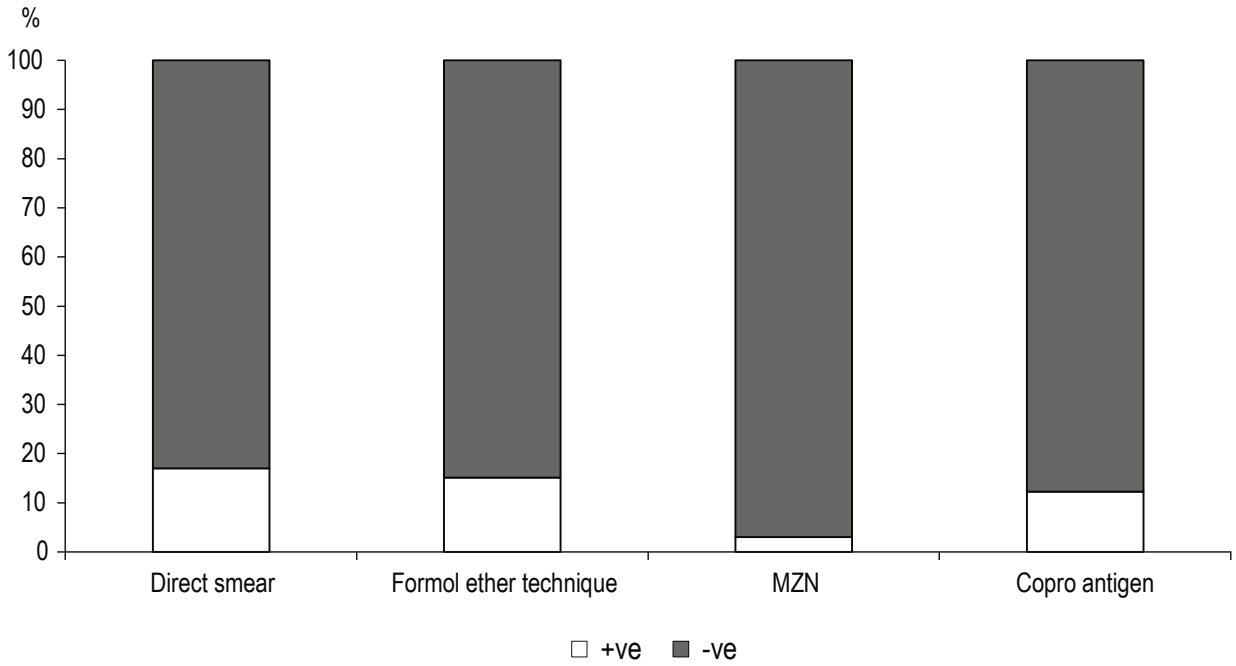

Fig. 3. Chart of diagnostic performance of different diagnostic techniques for stool examination.

adult age (19 – 60 years, 40 % of cases). In the school age group, there were 29 males and 10 females while in adult age, there were 17 males and 9 females. Among 16 parasitic infected cases, ten cases (62.5 %) were in school age. A significantly higher prevalence of parasitic infections was detected in males of this group (90 %) compared to females. While six cases (37.5 %) were in adult age with also increase in the prevalence of parasitic infections among male (66.6 %) of this group (Table1).

Regarding residence, Out of the 16 positive parasitic infected cases, there were 13 (20 %) from rural areas while three (4.6 %) from urban areas. Ten cases had history of diarrhea with or without blood. A single case from all studied patients had a history of *Schistosoma mansoni* infection.

Parasitological study

Parasitological examination of luminal content of removed appendices and histopathological examination of stained specimens revealed that 16 (24.6 %) positive cases from a total of 65 appendectomized patients had parasitic infections.

Out of the 16 positive parasitic infected cases, there were four cases had *Giardia duodenalis* cysts (25 %), four cases had *Entamaeba histolytica* cysts (25 %), two cases had *Cryptosporidium* spp. oocysts (12.5 %), two cases had *Hymenolepis nana* ova (12.5 %), one case had *H. diminuta* ova (6.25 %) and one case had *Blastocystis* spp. cysts (6.25 %) that were detected by examination of appendiceal faecolith (Figs. 1 and 2). Other two cases, *Schistosoma mansoni* ova (6.25 %) and *E. vermicularis* adult (6.25 %), were detected by histopathological examination of stained specimens.

Regarding different methods of stool examination used in diagnosis of parasitic infections, the number of positive parasitic cases detected by direct smear examination (17 %) was higher than those detected by formol-ether concentration technique (15.4 %), immunodiagnostic technique (12.3 %) or modified Ziehl-Neelsen (3.1 %). While the number of positive cases of *Cryptosporidium*

Table 4. Blood picture findings in school age group.

School age		Range	Mean ± S. D	t. test	p. value
Hb g/dl	Infected positive cases	8 – 12	10.10 ± 1.29	20.777	0.001*
	Negative cases	10 – 12	11.45 ± 0.57		
TLC /mm^3 of blood	Infected positive cases	8000 – 11000	10.4 ± 6.9	0.208	0.651
	Negative cases	1000 – 11000	10.3 ± 4.7		
Eosinophilic Count %	Infected positive cases	10 – 15	13.80 ± 2.04	46.152	0.001*
	Negative cases	6 – 8	8.00 ± 0.00		

* extremely significant

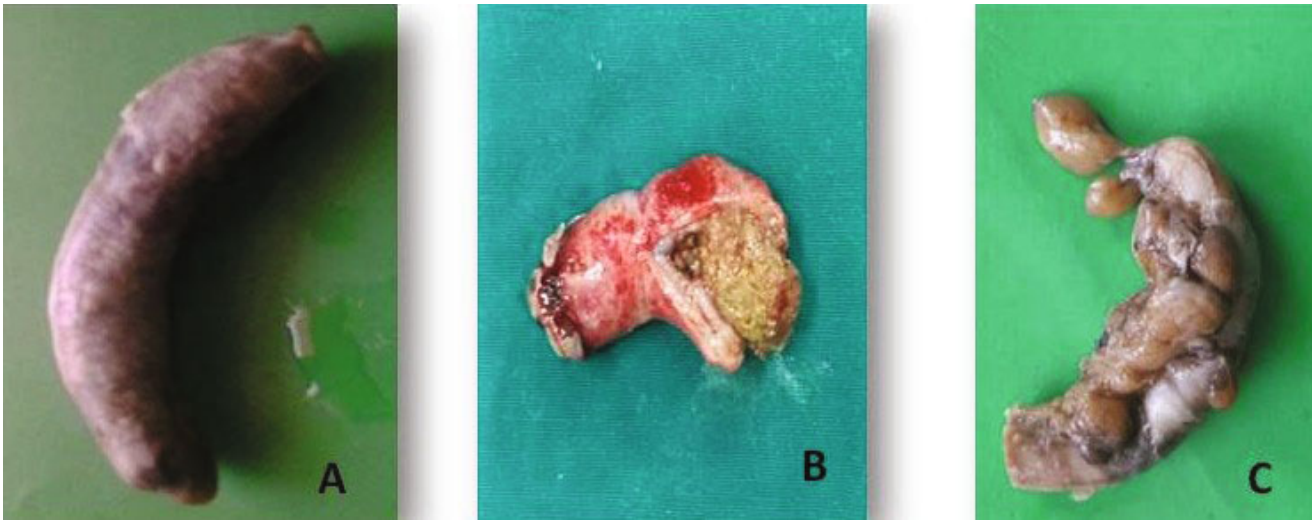

Fig. 4. Gross appearance of inflamed appendices showing: A: inflamed appendices removed for acute appendicitis showing swollen, dull serosa. B: congested appendix filled with faecolith. C: chronic appendicitis showing marked thickening in the wall and fat creeping on serosa.

spp. detected by modified Ziehl-Neelsen was higher than other methods (Fig. 3).

Using direct smear as the gold standard, the sensitivity of formol-ether concentration and immunodiagnostic techniques for *E. histolytica* detection were 100. While the sensitivity of formol-ether concentration and immunodiagnostic techniques for *G. duodenalis* detection were 75 and 100, respectively. The specificity of formol-ether concentration and immunodiagnostic techniques for *Cryptosporidium* was 100 (Table 3).

Laboratory blood picture findings

In this study complete blood picture was done. Regarding haemoglobin (HB) count in school age group, there was anemia in cases infected with parasites as their HB level ranged from 8 to 12 g/dl in appendectomized infected patients with a significant difference between infected and non infected cases. While in adult group there was no difference between infected and non infected cases. Leucocytic count in all age groups ranged from 8000 – 11000 with a significant difference between parasite-infected and non infected cases in adult age group. Concerning the eosinophilic count in all age groups, there was elevation of the count (10 – 15 %) in parasitic infected appendectomized cases in comparison to non infected cases with significant difference between them (Table 4 and 5).

Histopathological findings

Gross appearance of inflamed acute appendices showed swollen, dull serosa. Some of them are congested filled with faecolith. Moreover, subacute appendicitis cases showed marked thickening in the wall associated with fat creeping on serosa (Fig. 4). Histopathological features of acute inflammation of the appendix were evident in 44 (67.7 %) cases in the form of transmural infiltration by acute inflammatory cells, mainly polymophnuclear leucocytes (PNLs), pus cells and macrophages (Table 6). Fourteen cases (21.5 %) of acute appendicitis showed eosinophilic infiltration that was associated with parasitic infections. Moreover, there was one case of acute suppurative inflammation associated with *E. histolytica* infection as appeared by PAS stain that stained trophozoites red (Fig. 5).

Subacute appendicitis was represent in 19 (29.2 %) of the studied cases. These cases showed chronic inflammatory reaction associated with reactive follicular hyperplasia in Peyer's patches and lymphoplasmacytic infiltration of the appendix wall.

Table 5. Blood picture findings in adult age group.

Adult age		Range	Mean ± S. D	t. test	p. value
Hb g/dl	Infected positive cases	10 – 12	11.17 ± 0.75	1.142	0.296
	Negative cases	11 – 12	11.45 ± 0.51		
TLC /mm^3 of blood	Infected positive cases	9000 – 12000	10.5 ± 1.22	14.740	0.001*
	Negative cases	9000 – 11000	9.2 ± 5.23		
Eosinophilic count %	Infected positive cases	10 – 15	12.83 ± 2.23	75.391	0.001*
	Negative cases	6 – 8	6.10 ± 0.45		

* extremely significant

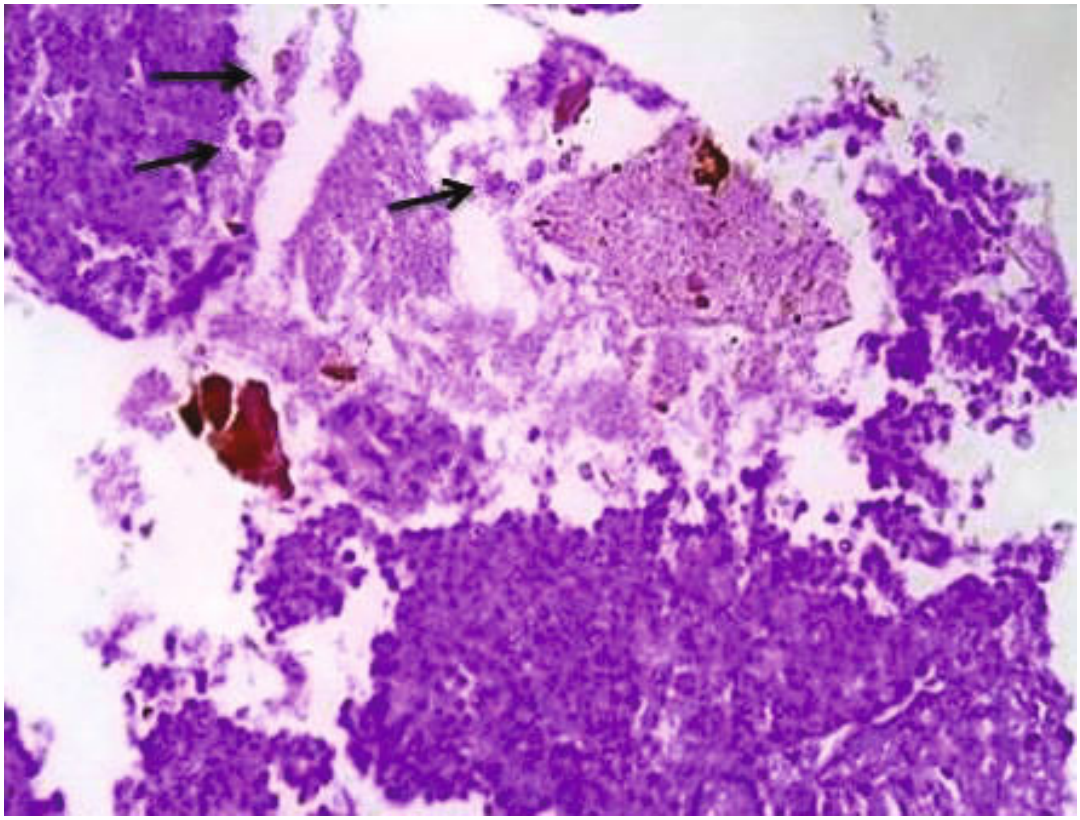

Fig. 5. Histopathological changes of resected appendices showing: acute suppurative appendicitis showing ulceration of the mucosa and amoeba trophozoites are found among pus cells in the lumen (arrows) (PAS stain x400).

Negative appendices (no acute inflammatory cellular reaction) were represented in 3.08 % of cases. One of them showed non gravid *E. vermicularis* adult female in the submucosa associated with mild eosinophilic infiltration and intact mucosa (Fig. 6A, B). Another case showed remnant of *S. mansoni* ova surrounded by a granuloma formed of epithelioid cells, eosinophils, lymphocytes, foreign body giant cells, fibroblasts and fibrous tissue (Fig. 6C, D).

Discussion

Appendicitis is considered a common cause of emergent abdominal surgical procedures. Moreover, epidemiologic studies have revealed that approximately 7 % – 12 % of the population would have appendicitis in their life time (Hardin, 1999; Mowlavi *et al*., 2004).

The aetiology of acute appendicitis has not been established and is still much debated. Several factors have been suggested which include diet, lymphoid hyperplasia, faecolith, and infections due to bacteria, viruses and parasites (Pasupati *et al*., 2008).

The association between parasitic infection of the appendix and acute appendicitis has been widely investigated. Therefore, this study was conducted to determine the prevalence of intestinal parasites in luminal content (faecolith) and tissue sections of appendectomy specimens in Tanta University Hospital, Egypt.

Parasitic infections, helminth and protozoa, were found in 16 (24.6 %) of cases in this study by feacolith and tissue sections examined. This percentage is close to reports of Pasupati *et al*. (2008) in Malaysia. On the other hand, Ebrahim (2010) reported the presence of parasitic infections in (57 %) appendix specimens in Alexandria, Egypt. Low incidences of parasitic infections were found in 5.5 % of appendices in Oman and Turky (Zakaria *et al*., 2013;Yabanoglu *et al*., 2014). Moreover, 9 % parasitic infections in appendix specimens were reported by Abdellatif *et al*. (2015) in Egypt.

Recently in Egypt, a retrospective study of appendectomies by Hedya *et al*. (2012) reported that 11 out of 251 specimens (4.38 %) had parasitic infections. In 2014, Jada *et al*. detected a wide spectrum of parasitic infections (48 %) in one hundred surgically removed appendices in India.

Out of the 16 positive specimens detected in this study by either faecolith or tissue sections examined, there were *Giardia lamblia* cysts (25 %), *E. histolytica* cysts and trophozoites (25 %), *Cryptosporidium* spp oocysts. (12.5 %), *H. nana* ova (12.5 %), *Schistosoma mansoni* ova (6.25 %), *E. vermicularis* adult (6.25 %), *H. diminuta* ova (6.25 %) and *Blastocystis* spp.cysts (6.25 %).

There was slight preponderance of males to females in this work. The percentage of parasitic infections was generally higher in males and this coincides with a recent study by Abdellatif *et al*.

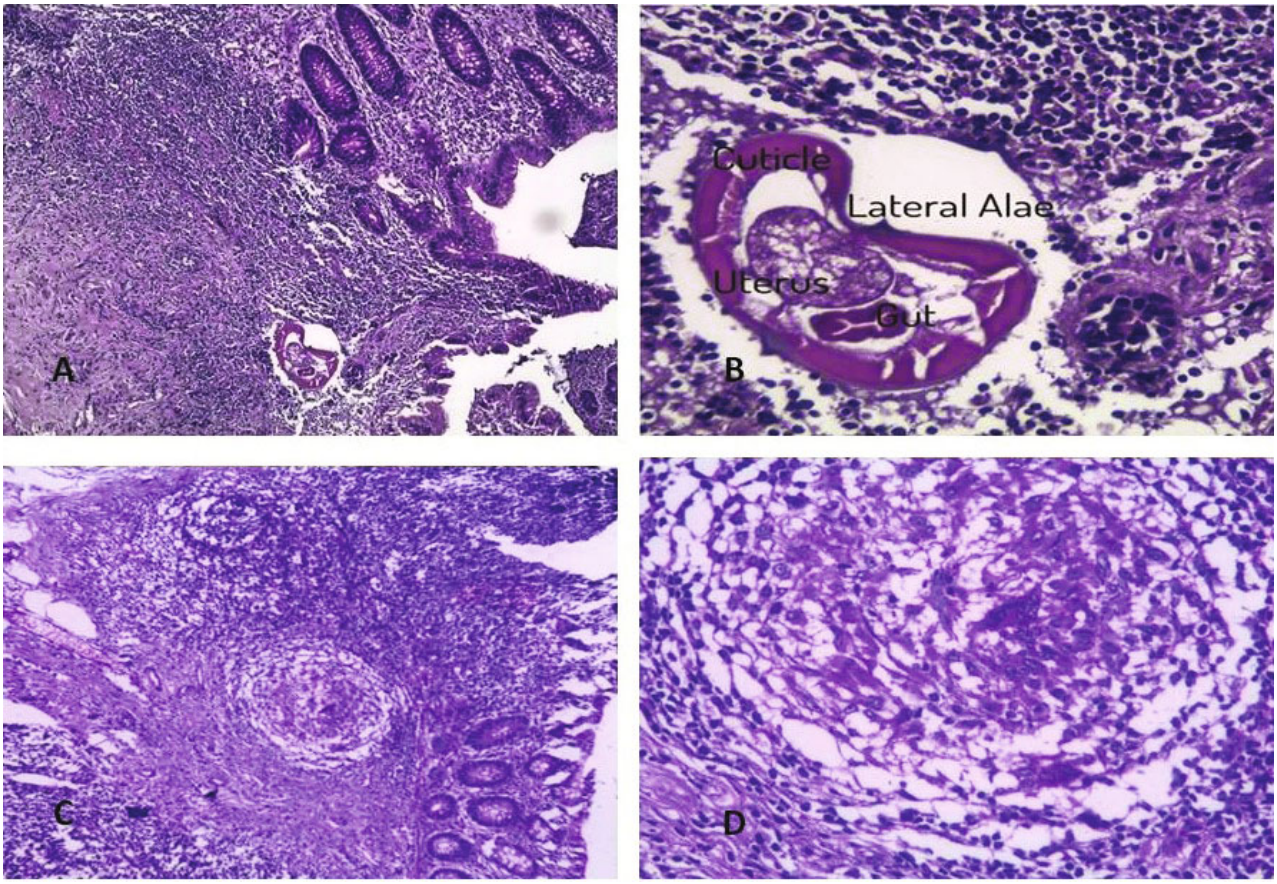

Fig. 6.: Histopathological changes of resected appendices (H&E) showing: A: non gravid adult female *E.vermicularis* in the submucosa of the appendix with inflammatory infiltrate and intact mucosa (x100). B: higher magnification of A showing characteristic lateral alae, the gut and the uterus of the worm with lymphoplasmacytic and eosinophilic inflammatory infiltrate surrounding it (x400). C: *Schistosoma mansoni* granuloma formation in the submucosa surrounding remenant of the ova (x100). D: higher magnification of C showing granuloma formed of epithelioid cells, lymphocytes and foreign body giant cells all surrounded by fibroblasts and fibrous tissue (x400).

(2015) who reported that the prevalence of infection, male: female ratio was 2.1: 1.2. On the contrary, Ahmed *et al*. (2015) mentioned that females are more vulnerable gender to be infected. However, no statistical significant difference was found between the rate of infections in males and females in his study.

Concerning age in this study, parasitic infections were higher in the age group ranging from 6 – 18 years. This finding is consistent with studies done by Pasupati *et al*. (2008) and Zakaria *et al*. (2013) who found infections in ages less than 22 years old. Therefore, there is a considerable range of reported prevalence of parasitic appendicitis in both children and adults, which likely reflects differences in parasite endemicity, demographic factors, and differences in diagnostic method (Hegazi and Patel, 2012)

This study was conducted in Gharbiya Governorate which is considered a rural -urban area (El-Khoby *et al*., 2000). Most of the parasites detected in the current work are transmitted by faeco-oral route and are widely spread in rural areas. This may explain the significant difference detected in infections according to the patients residence in this work.

These results coincide with the findings of other studies focused on rural areas around the world such as Tang and Luo (2003) in China, Çelİksöz *et al*. (2005) in Turkey, Ikeh *et al*. (2006) in Nigeria, Jacobsen *et al*. (2007) in Ecuador and Ngrenngarmlert *et al*. (2007) in Thailand, where the infection rate was peaking in comparison to the urban areas. The high rate is back to environmental factors, poor personal hygiene and lack of health education. Therefore, all patients are at risk of harboring intestinal parasites.

S. mansoni granuloma was found in one case with no acute inflammatory changes of appendiceal specimens were observed. This observation is in accordance with Duzgun *et al*. (2004) who found one case of *S. mansoni* infection and mentioned that the role of *S. mansoni* infection in appendicitis is doubtful. On the other hand, Abdellatif *et al*. (2015) mentioned that *Schistosoma* granuloma was important cause of appendicitis. Other reports mentioned that the actual role of *S. mansoni* infection in the development of appendicitis is still open to debate and has been the subject of controversy (Limaiem *et al*., 2015). Also, it is mentioned that schistosomal appendicitis occurred when obstruction of the lumen by long standing granulomatous reaction around *Schistosoma* ova occurred (Satti *et al*., 1987).

Table 6. Histopathological finding of appendix specimens.

Histopathologic type	Acute appendicitis		Subacute appendicitis		Negative appendices		Total	
	No.	%	No.	%	No.	%	No.	%
No. examined	44	67.7	19	29.2	2	3.08	65	100
No. Parasitic Infected cases	14	21.5	0	0	2	3.08	16	24.6
E.vermicularis	0	0	0	0	1	1.54	1	1.54
Schistosoma mansoni	0	0	0	0	1	1.54	1	1.54
G.duodenalis	4	6.15	0	0	0	0	4	6.15
E.histolytica	4	6.15	0	0	0	0	4	6.15
H.nana	2	3.08	0	0	0	0	2	3.08
H.diminuta	1	1.54	0	0	0	0	1	1.54
Cryptosporidium spp.	2	3.08	0	0	0	0	2	3.08
Blastocystis spp.	1	1.54	0	0	0	0	1	1.54
P-value					0.021*			

* significant

Also in the current research, one case of *E. vermicularis* in the studied appendectomy specimens (1.5 %) was found. This is nearly similar to previous report by Ahmed *et al*. (2015) who mentioned that occurrence of pinworms with appendicitis ranged between 0.2 – 41 % worldwide. Other studies reported the frequency of *E. vermicularis* in the appendices was 0.6 – 3.8 % depending on the geographic area of the studied cases (Isik *et al*., 2006; Sah and Bhadani, 2006; da Silva *et al*., 2007; Chamisa, 2009).

A unique finding in this study is the presence of *E. vermicularis* adult in the submucosa of the appendix with the characteristic morphological features (lateral alae), not in the lumen as it is used to be. Babady *et al*. (2011) mentioned that rarely the *E. vermicularis* adult worms could become lodged in the intestinal mucosa and cause abscess.

Worth mentioning, that there was only mild eosinophilic infiltration around the *Enterobius* worm in submucosa and no acute inflammation was detected in the appendix specimen containing *Enterobius* worm. This is in agreement with majority of studies which reported low incidence of inflammation with enterobiasis of the appendix (Sinniah *et al*., 1991; Wiebe, 1991; Panidis, 2011).

On the contrary, some studies found acute or chronic inflammation with pinworms in the lumen of the appendix (Dorfman *et al*., 1995; Saxena *et al*., 2001). It is justified to say that our results agreed with the reported cases worldwide that does not settle the controversy about the relation between *E. vermicularis* to appendicitis (Arca *et al*., 2004; Panidis *et al*., 2011).

On the other hand, some workers believed that pinworms manifest meticulously in the bowel according to some retrospective studies and it is the most common worm residing in the appendix, leading to pathological changes including inflammation, lymphoid hyperplasia, and subsequently complications like peritonitis and gangrene (da Silva *et al*., 2007). Keeping in mind those intestinal parasites may cause symptoms and signs similar to that of acute appendicitis; this may explain the normal histology of appendectomy specimen infected with *E. vermicularis* (Yabanoglu *et al*., 2014).

Likewise, some minor degree of lymphoid hyperplasia may follow parasitic infection and not always cause symptoms. Moreover, pathogenesis of acute appendicitis may be due to either inflammation secondary to presence of parasites or obstruction of the lumen of the appendix by the parasite (Dorfman *et al*., 1995).

In respect to *E. histolytica* in the appendix, it is quite rare and the exact incidence of this presentation is not well known (Sartorelli *et al*., 2005). In this work four cases were found (6.15 %). The literature contains only few reports of amoebic appendicitis. When it occurs, it usually develops as an extension of caecal infection (Nadler *et al*., 1990; Singh *et al*., 2010). This infection should be considered in the treating physician's mind (Ito *et al*., 2014). Aggregated trophozoites in the cecum and small intestine after appendectomy are likely and require treatment. Therefore, the use of antibiotic combined with anti-amoebic metronidazole post-operative therapy will reduce the incidence of complications especially in high risk regions such as Egypt.

Of interest, *H. nana* and *H. diminuta* ova were found in four faecolith samples. This is a novel finding and their presence could be a coincidental finding with doubtful role in pathogenesis of appendicitis.

Moreover, *Blastocystis hominis*, infection was diagnosed in one case in this study. This is in accordance with Pasupati *et al*. (2008) who detected *B. hominis* in appendicial luminal content with *Cryptosporidia* and *Microsporidia*. *Blastocystis* has been reported to invade the intestinal lamina propria in humans leading to inflammation (Al-Tawil *et al*., 1994). In literature, only few cases were recorded in the appendix, therefore its role in appendicitis is questionable.

Giardia duodenalis (25 %) and *Cryptosporidium* (12.5 %) were also found in luminal content and in stool samples of parasitic infected appendectomized patients in this study.

Cryptosporidial enteritis may mimic acute appendicitis (Amer *et al*., 2016). They reported a patient presenting with right iliac fossa pain and diarrhea and was operated for appendectomy. This appendix was grossly normal and stool samples revealed *Cryptosporidia* oocysts which was the cause of false appendicitis.

The question is whether presence of parasites in the specimens is incidental or a factor of inflammation. Aydin (2007) mentioned that an appendix hosting parasites may suffer from primary or secondary inflammation in the appendix as it has narrow lumen.

The surgeon, handling a suspected case of appendicitis, should be aware that parasites may cause colic similar to appendicitis. Therefore, eosinophilic count and rapid examination of stool samples should be done before surgery especially in children (Fleming *et al*., 2015). This work sheds light on the differential diagnosis and treatment plans for future cases with abdominal colic similar to that of acute appendicitis.

The rate of parasitic infections detected in the study group arouse the possibility of some of them may have contracted infection in hospital. According to Lettau (1991), parasitic diseases are generally due to lack of cleanliness in the rooms, bathrooms and kitchens in the hospitals. Similarly, El-Sibaei (2003), reported that 8 % of patients developed diarrhea after admission to Ain-Shams pediatric hospital in Egypt and they were found to be positive for cryptosporidiosis.

Regarding eosinophilia detected in the studied patients it was distributed as follows: 6 % in negative patients for parasites, 15 % in cases with parasitic infection. Although most of protozoa infections seldom cause eosinophilia, the eosinophil count was significantly higher with protozoa infections in patients originally suffering from other disease. As regards enterobiasis, Seybolt *et al*. (2006) reported that it wouldn't cause eosinophilia unless the parasite invades tissues, as reported in our study.

The pathological examination of tissue sections in this study showed a range of findings. Acute inflammation was found in (67.69 %) of cases, with neutrophil infiltration or neutrophil and eosinophil infiltration. Two cases of them were diagnosed as periappendiceal abscesses, chronic inflammatory reaction with lymphoid hyperplasia was found in (29.23 %) which may initiate the start of inflammatory process. Two cases (3.08 %) showed no acute reaction and were considered as negative appendices.

In this study, immunochromographic test for *E. histolytica*, *G. duodenalis* and *C. parvum* copro-antigen detection was selected as a rapid test for stool examination and its performance was compared with microscopic examination as gold standard. This test is recommended for diagnosis of protozoa infection because of simplicity of use. There was no evidence of cross reactivity using the kit with other parasites identified in the stool specimens and can be used for screening purposes in large scale studies or outbreak investigations or as a possible alternative to stool examination (Swierczewski *et al*., 2012).

In this study, results of immunodiagnostic test for detection of copro-antigen show sensitivity of 100 % for *E. histolytica* and 100 % for *G. duodenalis*. *E. histolytica* sensitivity was found to be 54.5 % in the work of Gatti *et al*. (2002). Regarding cryptosporidial infection, the specificity of immunodiagnostic technique was 100 and it needs further evaluation since it failed to detect any positive cases. This may be due to low oocyst density. Also, may be due to genetic diversity of *Cryptosporidium* (Goni *et al*., 2012). Salman (2014) found that direct fluorescent assay and modified Ziehl-Neelsen methods show high sensitivity and specificity in *Cryptosporidium* diagnosis than ELISA-corpo antigen. On the other hand, Hawash (2014) recommended this test as rapid and highly sensitive and specific test.

This study has shed light on the problem of patients in surgical ward as being source for spread of parasitic diseases. Many types of food borne intestinal parasitic infections were found in these patients, and intestinal protozoa were of special importance.

Therefore, it is recommended to consider the possibility of parasitic infections in these patients in order to reduce the associated co morbidity and suffering. Also detection of copro-antigens should be included in the laboratory diagnostic work-up for parasitic infections in these patients.

Finally, health education and implementation of infection control measures will always be advisable. It is recommended that routine histopathological examination of appendiceal specimens should be done for diagnosing unsuspected conditions such as parasitic infections that require further treatment.

In conclusion, parasitic infections were detected in 24.6 % of appendictomized patients at Tanta University Hospitals, Egypt. Most of parasitic infections were prevalent in patients belonging to the school age group. So, intestinal parasites may cause symptoms and signs similar to that of acute appendicitis. Therefore, careful attention to clinical history, stool examination and high eosinophilia may aid diagnosis and avoid unnecessary appendectomy. The presence of some ova and parasites in the narrow lumen of the appendix may be a co-factor in the development of appendicitis and this needs further studies. Rapid examination of stool samples and eosinophilic count should be done in case of appendiceal colic in endemic areas to diagnose intestinal parasites. The use of antibiotic combined with antiamoebic post-operative therapy will reduce the incidence of complications especially in high risk regions as Egypt. Immunodiagnostic test for the detection of copro-antigen was a successful rapid diagnostic technique, but still needs further evaluation in more cases of cryptosporidial infection, especially when stool samples were microscopically positive.

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Helminth fauna of Spiny Tailed Lizard, *Darevskia rudis* (Bedriaga, 1886) (Sauria: Lacertidae) from Turkey

S. BİRLİK[1], H. SAMİ YILDIRIMHAN[1], Ç. ILGAZ[2,3], Y. KUMLUTAŞ[2,3]

[1]Uludağ University, Faculty of Arts and Sciences, Department of Biology, Nilüfer, Bursa, Turkey, *E-mail: *sezen@uludag.edu.tr*;
[2]Dokuz Eylül University, Faculty of Science, Department of Biology, 35160, Buca-İzmir, Turkey;
[3]Dokuz Eylül University, Fauna and Flora Research Centre, 35610, Buca-İzmir, Turkey

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Summary

The present study investigated the composition of helminth parasites of *Darevskia rudis,* Spiny Tailed Lizard from Turkey. One hundred and two samples (49♀♀, 53♂♂) from the Tokat, Trabzon, Rize, Gümüşhane and Artvin Provinces were collected and examined for helminth parasites. New host and locality records were recorded. As a result of the present study, seven species of Nematoda, *Skrjabinodon medinae*, *Spauligodon* sp., *Spauligodon carbonelli*, *Spauligodon aloisei*, *Skrjabinelazia hoffmanni*, *Strongyloides darevsky*, *Oswaldocruzia filiformis*; one species of Cestoda, *Mesocestoides* spp. (tetrathyridium) and one species of Acanthocephala *Sphaerirostris scanensis* were reported from the lizard samples. *Sp. carbonelli* and *Sphaerirostris scanensis* are here recorded for the first time in Turkey. *D. rudis* is the new host recorded for *Sk. medinae*, *Spauligodon* sp., *Sp. carbonelli*, *Sp. aloisei* from Nematoda, *Mesocestoides* spp. from Cestoda and *Sphaerirostris scanensis* from Acanthocephala. This host has been studied for the first time for the helminth parasites from the Tokat and Gümüşhane Provinces. There is, to our knowledge, only one report of helminthes for *D. rudis* in Turkey.

Keywords: *Darevskia rudis,* Helminth, Cestoda, Nematoda, Acanthocephala, Turkey

Introduction

Caucasian rock lizards (*Darevskia* spp.) are small lacertids from western Asia and south-eastern Europe. *Darevskia rudis*, Spiny Tailed Lizard, (Bedriaga, 1886) is a common species which occurs in Turkey (including northern Anatolia and the Middle Taurus Mountains), Georgia, Russia and Azerbaijan. This species ranges from sea level to 2400 m a.s.l., inhabiting rocky areas in temperate forests but it may also occurs in montane-steppe habitats and on the walls of buildings and other human structures (Baran *et al*., 2012; Arribas *et al*., 2013).

Both domestic and wild animals are hosts of a wide variety of parasitic species therefore some helminth parasites studies have been carried out in our country, especially for lacertid lizards. In Turkey, there are 39 species of lizards belonging to family Lacertidae. The species which have been studied for their helminth fauna are: *Lacerta viridis* (Schad *et al.,* 1960), *Podarcis tauricus* (Schad *et al.,* 1960), *Parvilacerta parva* (Saygı & Olgun, 1993), *Anatololacerta danfordi* (Gürelli *et al.,* 2007), *Lacerta trilineata* (Yıldırımhan *et al.,* 2011), *Eremias pleskei, E. strauchi*, *E. suphani* (Düsen *et al.* 2013), *Apathya cappadocica* (Birlik *et al.,* 2015), *Darevskia rudis* (Roca *et al.,* 2015a), *Darevskia uzzelli*, *D. bendimahiensis* and *D. sapphirina* (Roca *et al.,* 2015b), *D. clarkorum, D. raddei, D. parvula, D. valentini, D. armeniaca, D. unisexualis* (Roca *et al.,* 2016), *Phoenicolacerta laevis* (Birlik *et al.,* 2016), *Acanthocadtylus harranensis, A. schreiberi, Mesalina brevirostris* (Düşen *et al.,* 2016) and *Iranolacerta brandtii* (Birlik *et al.,* 2017).

To our knowledge, there is only one report of specific helminths

* – corresponding author

in *Darevskia rudis*. The first study was conducted by Roca *et al.* (2015a). This is the second helminth study of this host in Turkey. Our objective is to gather information about the *D. rudis* helminth fauna and report on the differences between helminth species and present new locations of occurrence for the parasites.

Material and Methods

We examined 102 *D. rudis* specimens, of which 49 were adult females (SVL: 42.6 ± 2.3), and 53 adult males (mean snout-vent length: 57.85 ± 9.25 with a range from 31.61 to 82.98). Lizards were captured by hand from different localities of the Tokat, Trabzon, Rize, Gümüşhane and Artvin Provinces (Fig. 1). Lizard specimens were anesthetized with ether, fixed with a 96 % ethanol injection, and deposited in 96 % ethanol. The body cavity of the lizards was opened, and the digestive tract removed. The oesophagus, stomach, small and large intestine and lungs were opened and examined for helminths under a dissecting microscope. The Nematodes were killed in hot saline solution, fixed in 70 % ethanol, and mounted in glycerol. For the morphological examination, the helminth species were cleared gradually in glycerin. Cestodes were fixed in 70 % ethanol, stained with iron-carmine as described by Georgiev *et al*. (1986), cleared in clove oil, and mounted in Entellan. The parasites were identified, when possible, to species, and the number and location of the individuals of each species were recorded. Helminth identification was based on keys given by Yorke and Maplestone (1926), Schmidt (1986), Yamaguti (1961,1963), Baker (1987), Petter and Quentin (1976), Anderson (2000). Helminth voucher specimens were deposited in the Uludağ University, Department of Biology, Bursa, Turkey; lizard specimens were deposited in the Dokuz Eylül University, Department of Biology, İzmir, Turkey. Prevalence, mean intensity and mean abundance were determined according to Bush *et al.* (1997). Specifically, prevalence is the percentage infected of individuals, mean intensity is the estimated number of parasites per infected individual, measured in specimens per infected animal and finally mean abundance is the estimated number of parasites per total number of examined host, measured as specimens per analysed animal.

Results

Fifty-six lizards were infected (55 %) and nine species of helminths were identified (Table 1): seven species of Nematoda, *Skrjabinodon medinae*, *Spauligodon* sp., *Spauligodon carbonelli*, *Spauligodon aloisei*, *Skrjabinelazia hoffmanni*, *Strongyloides darevsky*, *Oswaldocruzia filiformis*; one species of Cestoda, *Mesocestoides* spp. (tetrathyridium) and one species of Acanthocephala *Sphaerirostris scanensis*. In total 175 individuals of nine parasite species were collected from 56 of the 102 Spiny-Tailed Lizards examined.

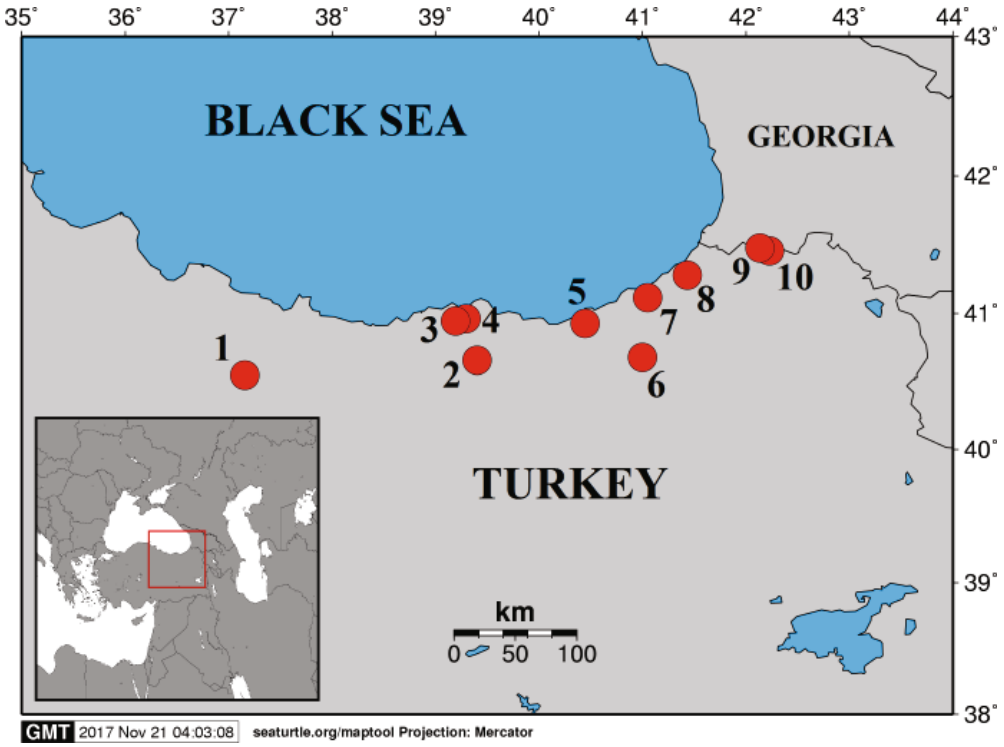

Fig. 1. The localities of host populations of *Darevskia rudis* (1. Karacaören, Başçiftlik, Tokat; 2. Zigana Pass, Gümüşhane; 3. Şalpazarı, Trabzon; 4. between Tonya and Vakfıkebir 10. km, Trabzon; 5. Kalkandere, Rize; 6. Ovit Pass, Rize; 7. Köprüköy, Rize; 8. Dülgerli Village, Ortacalar, Arhavi, Artvin; 9. Papart Plateau; 10. Meydancık Village, Şavşat, Artvin

There were nine helminth species represented in the lizards but no individual host harbored more than three helminth species. Of the infected lizards, 40 harbored one species of helminth, 15 harbored two species, one harbored three species. In this study, *D. rudis* is the new host record for *Sk. medinae*, *Spauligodon* sp., *Sp. carbonelli*, *Sp. aloisei* from Nematoda, *Mesocestoides* spp. from Cestoda and *Sphaerirorstris scanensis* from Acanthocephala. This host has been studied for the first time for helminth parasites from Tokat and Gümüşhane provinces. *Sp. carbonelli* and *Sphaerirostris scanensis* are recorded for the first time in Turkey. Prevalence, mean intensity and mean abundance of helminth species found in lizards were given in Table 1.

Table 1. Prevalence, mean intensity and mean abundance.

Helmint Species	Site of infection	Prevalence (%)	Mean intensity	Mean abundance
Nematoda				
Skrjabinodon medinae	Small intestine	33.33	2.2	0.73
Spauligodon sp.	Small intestine	4.9	2	0.09
Spauligodon carbonelli	Small intestine	9.8	2.2	0.21
Spauligodon aloisei	Small intestine	2.94	1.33	0.03
Strongyloides darevsky	Small intestine	10.78	4.18	0.45
Skrjabinelazia hoffmanni	Small intestine	0.98	1	0.009
Oswaldocruzia filiformis	Small intestine	1.96	2.5	0.04
Acanthocephala				
Sphaerirostris scanensis	Small intestine	1.96	1	0.019

Discussion

This study is the second helminth study on *D. rudis* specimens inhabited in Turkey. The First study was conducted by Roca *et al.* (2015a) on lizard specimens from the Ardahan, Artvin, Rize, Trabzon, Bursa, Düzce, Zonguldak, Karabük, Bolu and Bartın Provinces. In that study, one cestode *Nematotaenia tarentolae* and four nematode species, *Spauligodon saxicolae*, *Skrjabinelazia hoffmanni*, *Oswaldocruzia filiformis* and *Strongyloides darevskyi* were reported.

In the present study, specimens collected from Tokat, Trabzon, Rize, Gümüşhane and Artvin Proviences were studied. We found additional helminth species. These are *Sk. medinae*, *Sp. carbonelli*, *Sp. aloisei*, one species of Cestoda, *Mesocestoides* spp. (tetrathyridium) and one species of Acanthocephala, *Sphaerirostris scanensis*. Also, one species of Nematoda was identified as genus level *Spauligodon* sp.

In both studies, only three nematode species have been found in common. These species are *Strongyloides darevsky*, *Skrjabinelazia hoffmanni* and *Oswaldocruzia filiformis*. At present, *S. darevsky* is a *Darevskia* specialist. *Strongyloides* spp. have been recorded in different reptile hosts worldwide, including the species *S. cruzi* Rodrigues, 1968, *S. ophiusensis* Roca & Hornero, 1992 and *S. darevskyi* Sharpilo, 1976 described from lizards (Rodrigues, 1968, 1970; Sharpilo, 1973, 1976; Roca & Hornero, 1992; Khanum *et al*., 2002; Murvanidze *et al*., 2008). Moreover, unidentified *Strongyloides* spp. have also been reported in these reptiles (Perera *et al*., 2013). Although many species of *Strongyloides* show a low intensity of infection, higher prevalences of *S. darevskyi* (up to 75 %) with variable intensities of infection (1 – 55) were found in some populations of *Darevskia* spp. from Transcaucasia (Sharpilo, 1973, 1976). In addition to the type host *D. saxicola* (Eversmann, 1884), *S. darevskyi* was reported in *D. armeniaca* (Méhely, 1909), *D. rostombekovi* (Darevsky, 1957) and *D. rudis* (Bedriaga, 1886). This is the third record of this species in Turkey. The first one was reported in *D. rudis* by Roca *et al.* (2015a), the second is from *D. armeniaca* by Roca *et al*. (2016).

In Turkey, reports of *Strongyloides* spp. in lizards are scarce. This species was reported from two host in Turkey: *D. rudis* (Roca *et al.* 2015a) and *D. armeniaca* (Roca *et al*. 2016). *Strongyloides darevskyi* is in fact a true *Darevskia* specialist since it has been recorded only in species of this genus (Roca *et al*., 2016).

Skrjabinelazia Sypliaxov, 1930 (Seuratidae: Skrjabinelaziinae) (see Chabaud, 1978) is a rarely reported nematode genus (Baker, 1987) which is parasitic in the insectivorous saurians, the Gekkonidae and Lacertidae, and exceptionally in the Iguanidae (see Freitas, 1940), Scincidae (see Goldberg & Bursey, 1995) and Gerrhosauridae (see Caballero, 1968). Males of *Skrjabinelazia* are smaller than females and their lifespan is probably shorter; they are often scarce compared to females (Chabaud *et al*., 1988; Freitas, 1940). The nematode *S. hoffmanni* is a generalist species that has been recorded in some genera of Palaearctic lacertid lizards, such as *Podarcis*, *Darevskia* and *Lacerta*. This is the third record of *S. hoffmanni* in Turkey, other reports are from *Anatololacerta danfordi* (Gürelli *et al.,* 2007) and *D. rudis* (Roca *et al.,* 2015a).

Oswaldocruzia filiformis belongs to the Order Strongylida, Family Molineidae: Head with cuticular vesicles, cuticle with transverse striations and longitudinal ridges, mouth with indistinct lips and a short esophagus. In the present study, both female and male specimens were found. This is the fifth report of *O. filiformis* in Turkey. Other records: *Lacerta viridis* and *Podarcis tauricus*, (Schad *et al*., 1960); *Anguis fragilis* (Düşen *et al.,* 2010), *Lacerta trilineata,*

(Yıldırımhan *et al.,* 2011) and *Darevskia rudis* (Roca *et al.,* 2015a). In addition to these common species, we also found some helminth species of Nematoda – *Skrjabinodon mediane*, *Spauligodon aloisei*, *Sp. carbonelli* and *Spauligodon* sp.

Skrjabinodon medinae lacks caudal alae and possesses a single pair of sessile pre-cloacal papillae. This is the fourth report of this species from our country. Others: *Lacerta trilineata* (Yıldırımhan *et al.,* 2011), *Apatyha cappadocica* (Birlik *et al.,* 2015) and *Phoenicolacerta laevis* (Birlik *et al.,* 2016).

Species of *Spauligodon* are separated on the basis of the presence or absence of a spicule, the tail filament morphology, egg morphology, and geographical distribution. Currently, 52 species are assigned to *Spauligodon* (Bursey *et al.* 2014a; Pazoki & Rahimian 2014). Based upon the zoogeographic regions described by Holt *et al.* (2013), 16 species is known from the Palaearctic realm. *Spauligodon aloisei* is the only species from the Palaearctic region which has prebulbar vulva position among all *Spauligodon* species. Previously, this species was reported from *Iranolacerta brandthii* (Birlik *et al*. 2017). This study is the second record of the species in Turkey.

The nematode *S. carbonelli* has been only recorded in the lizards *Podarcis muralis*, *P. carbonelli* and *P. hispanica* from the Iberian Peninsula (García-Adell & Roca, 1988; Roca *et al.,* 1989; Galdon *et al.,* 2006), it may be considered a *Podarcis* specialist. Many *Spauligodon* spp. Found in Palaearctic region do not have a spicule. Only 5 of 22 species have spicule and *Sp. carbonelli* has a shorter (15 – 35 µm) spicule than other male specimens with a spicule (40 – 70 µm). Our male specimens have a short spicule (22 – 28 µm) and a spiny tail (1 – 5) and the females show postbulbar vulva. *Sp. carbonelli* is recorded for the first time in Turkey.

We found both *Spauligodon* and *Skrjabinodon* species. This correlates with the usual pattern found in different European lacertid lizards, in which other *Spauligodon* species frequently share reptile hosts with members of the genera *Skrjabinodon* (but see Jorge *et al*., 2014) and *Parapharyngodon* (García-Adell & Roca, 1988; Roca *et al*., 1986, 2009; Roca & Hornero,1994).

Spauligodon saxicolae was found in a helminth study of *D. rudis* by Roca *et al.* (2015a) but we did not find this species in our lizard material. It is a nematode species of the Palaearctic region which have smooth tails in both male and female however males have no spicule. All of our nematode specimens found in this study have spiny tail and show spicules.

Mesocestoides spp., tetrathyridia are large, solid-bodied cysticercoids known only from the cyclophyllidean genus *Mesocestoides* are typically encountered embedded in the livers or coelomic mesenteries of their paratenic hosts. The genus has a worldwide distribution and is known from a great variety of amphibians and reptiles. It has been reported in the families Agamidae, Anguidae, Chamaeleonidae, Gekkonidae, Lacertidae and Scincidae (Witenberg, 1934; Hughes, 1940).The life cycle of species of *Mesocestoides* is believed to require 3 hosts: a vertebrate definitive host, a vertebrate second intermediate host, and an arthropod first intermediate host (Rausch, 1994). According to Specht and Voge (1965), the liver is the principal organ for natural infections in lizards. All the specimens found in this study were seen the liver. Tetrathyridia of the cyclophyllidean cestode, *Mesocestoides* sp. was reported previously from various lizards in Turkey *Anatalocerta danfordi* (Gürelli *et al.,* 2007), *Lacerta trilineata* (Yıldırımhan *et al.,* 2011), *Apatya cappadocica* (Birlik *et al.,* 2015), *Phoenicolacerta laevis* (Birlik *et al.,* 2016). This is the fifth report from lizards in Turkey.

Darevkia spp. show a lower prevalence of infection than the other continental Palaearctic rock lizards of the genus *Podarcis*. Roca *et al*. (2015a) associated these findings with the ecological characteristics of hosts and environment than in terms of the phylogeny of the lizard hosts.

***Sphaerirostris scanensis* (Lundström, 1942) Khokhlova, 1986,**

This species was recorded by Lundström (1942) from *Turdus merula* L., 1758 in Sweden. Comparing the morphometric data of the studied specimen with the original description, we found that the present species has a greater number of longitudinal rows of hooks – 28 versus 22 in the original description (Lundström, 1942) while the number of hooks per row and the morphology of the hook roots are comparable with those of *S. scanensis*. Another very similar species is *Sphaerirostris turdi* (Yamaguti, 1939) Golvan, 1956 described from thrushes and ortolans in Japan. The proboscis armament of this species consists of 26 – 34 longitudinal rows of hooks with 11 – 14 hooks per row (Yamaguti, 1939) and 26 rows x 11 – 13 hooks per row according to Kugi (1988). Velikanov (1989) recorded encysted juveniles (larvae) of this species from Lichtenstein's toadhead agama (*Phrynocephalus interscapularis* (Lichtenstein, 1856)) in Turkmenistan. However there aren't any reports of this species in Turkey.

56 of 102 (55 %) were infected by several helminth species but when the number of host specimens (102) was taken into consideration, the actual number of helminth (175) found were less. The biodiversity of helminth species was higher than in the study by Roca *et al.* (2015). These differences may be derived from season. Our lizard samples were collected in July and August. Samples studied by Roca *et al.* (2015a) were collected in spring. In particular, herbivorous reptiles are infected by different Pharyngodonidae than carnivores. Petter and Quentin (1976) recognized two evolutionary lineages (each one of them with different genera) parasitising carnivorous or herbivorous reptiles. Moreover, the structure of helminth communities is richer and more diverse in herbivorous reptiles (Roca and Hornero, 1992). It has been suggested (see Roca 1999) that the monoxenous life cycle typical of the Pharyngodonidae favours the infection of herbivorous reptile hosts because they have more opportunities to accidentally eat eggs deposited in plants through faecal pellets. Moreover, the increase of plant matter consumed provides a suitable environment for the development of a more rich and abundant helminth fauna (Petter and Quentin, 1976; Roca, 1999; Roca *et al.* 2005).

Several aspects of the biology and the ecology of lizards such as,

foraging modes, uses of microhabitats, body sizes, pregnancy, diet composition and ontogeny (Aho, 1990; Poulin, 1997) play a key roles in the acquisition of the associated helminth fauna. 13 *Darevskia* species inhabits in Turkey. Additional helminth studies are required to identify new helminth species, correlate the results with host characteristics and present new locality records. In summary, two new helminth records, six new host records and new geographic locality records are documented in this study.

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Optimum sample size to estimate mean parasite abundance in fish parasite surveys

S. SHVYDKA[1], V. SARABEEV[2]*, V. D. ESTRUCH[3], C. CADARSO-SUÁREZ[4]

[1]Department of Mathematics, Zaporizhzhia National University, Zhukovskogo 66, 69063 Zaporizhzhia, Ukraine; [2]Department of Biology, Zaporizhzhia National University, Zhukovskogo 66, 69063 Zaporizhzhia, Ukraine, *E-mail: *vosa@ext.uv.es*, *volodimir.sarabeev@gmail.com*; [3]Research Institute for Integrated Management of Coastal Zones, Department of Applied Mathematics, Polytechnic University of Valencia, Paranimf, 1, 46730 Grau de Gandia, Spain; [4]Unit of Biostatistics, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), University of Santiago de Compostela, Av. Barcelona, 15782 Santiago de Compostela, Spain

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Summary

To reach ethically and scientifically valid mean abundance values in parasitological and epidemiological studies this paper considers analytic and simulation approaches for sample size determination. The sample size estimation was carried out by applying mathematical formula with predetermined precision level and parameter of the negative binomial distribution estimated from the empirical data. A simulation approach to optimum sample size determination aimed at the estimation of true value of the mean abundance and its confidence interval (*CI*) was based on the Bag of Little Bootstraps (BLB). The abundance of two species of monogenean parasites *Ligophorus cephali* and *L. mediterraneus* from *Mugil cephalus* across the Azov-Black Seas localities were subjected to the analysis. The dispersion pattern of both helminth species could be characterized as a highly aggregated distribution with the variance being substantially larger than the mean abundance. The holistic approach applied here offers a wide range of appropriate methods in searching for the optimum sample size and the understanding about the expected precision level of the mean. Given the superior performance of the BLB relative to formulae with its few assumptions, the bootstrap procedure is the preferred method. Two important assessments were performed in the present study: i) based on *CI*s width a reasonable precision level for the mean abundance in parasitological surveys of *Ligophorus* spp. could be chosen between 0.8 and 0.5 with 1.6 and 1x mean of the *CI*s width, and ii) the sample size equal 80 or more host individuals allows accurate and precise estimation of mean abundance. Meanwhile for the host sample size in range between 25 and 40 individuals, the median estimates showed minimal bias but the sampling distribution skewed to the low values; a sample size of 10 host individuals yielded to unreliable estimates.

Keywords: fish; *Ligophorus* spp.; mean abundance; optimum sample size; precision; Bag of Little Bootstraps

Introduction

The mean abundance is the most common epidemiological index that quantifies parasites in host samples (Rózsa *et al*., 2000). In many cases, it is useful to know the optimum sample size in order to obtain the population's actual characteristics and their confidence intervals (*CI*s). The estimated parasitological indices are often based on small sample sizes due to high time or monetary constrains, logistical problems associated with host capture or low abundance of some host populations. On the other hand, if the sample size is too large, researchers' additional time, money and other resources might be a wasted effort for minimal gain. Statisti-

* – corresponding author

cal descriptors of fish parasites were calculated based on samples from three individuals to over 1000 (Poiani, 1992; Belghyti *et al*., 1994; Ismen & Bingel, 1999), but it was commonly done without special attention to the effect of sample size on these estimates.

In case of natural infections, parasites typically exhibit an aggregated distribution pattern, with most host individuals harbouring low numbers of parasites and a few individuals hosting too many (Anderson & Gordon, 1982; Shaw & Dobson, 1995; Poulin, 2013). In most cases, the aggregated distribution of the parasites can be fitted to the negative binomial distribution (NBD), where smaller values of the dispersion parameter k ($k < 1$) indicate a highly aggregated distribution attributed to the most macroparasites of wildlife hosts (Shaw & Dobson, 1995). The aggregated nature of parasite distribution affects the mean abundance and the width of its *CI* (Rózsa *et al*., 2000). Therefore, in the determination of the optimum sample size, a compromise between representativeness of the parasitological data from small samples and unnecessary costs for collection excess data should be found. In addition, the determination of the threshold for minimum sample size depends from level of precision that should be well understood and chosen by the researchers. A few studies have focused on the effect of sample size on parasitological parameters (Gregory & Woolhouse, 1993, Jovani & Tella, 2006, Marques & Cabral, 2007). Gregory and Woolhouse (1993) claimed that if parasite sampling is not correctly selected, it may result in artefactual patterns for epidemiologic and aggregation indices. Jovani and Tella (2006) argued that a sample size around 15 fish specimens is enough to get statistically acceptable data for estimating the actual prevalence within a population. Marques and Cabral (2007) examined the effects of sample size on estimates of infection indices and demonstrated that even though samples with less than 40 individuals do not substantially influence parasite prevalence, whereas the mean intensity and mean abundance may be underestimated. Thus, sample size determination is a common problem when dealing with parasitological data.

Karandinos (1976) presented a formula for sample size calculation with precision as a fixed proportion of the mean. The formula has been subsequently developed by Ruesink (1980) for data with distribution patterns ranging from highly clumped to uniform. However, these formulae are based on several assumptions that are often not fulfilled by actual data. An alternative approach to estimating the sample size is to use Monte Carlo simulations and bootstrapping techniques (Efron & Tibshirani, 1993).

The purpose of the present study is to explore the best method to determine the optimum sample size in parasitological surveys to obtain the true values of the mean abundance and their *CI*s. Marques and Cabral (2007) showed the power of Monte Carlo simulation and bootstrap procedures in determining the minimum sample size to estimate the mean abundance, mean intensity and prevalence. However, their work does not address the issue of parasite aggregation in the hosts. Moreover, the precision level and *CI*s for the studied indices were not considered, whereas these aspects are deliberately addressed here. The *CI* can provide information about estimation accuracy of the parasitological indices, where its width is used as a measure of estimation uncertainty and is largely determined by sample size. Marques and Cabral (2007) constrained their work to demersal flatfish and involved two species of cestodes, one acanthocephalan and one copepod. The present study focuses on monogenean parasites of *Ligophorus* spp. from the pelagic flathead grey mullet, *Mugil cephalus* L.

We considered two approaches, analytic and simulation, to estimate an adequate sample size to obtain ethically and scientifically valid values of mean abundance. To achieve this aim, the study is organized as follows. On the first stage, the required sample size was determined using a formula with predetermined precision levels. The second stage involved a simulation study based on applying the Bag of Little Bootstraps (BLB) (Kleiner *et al*., 2014) to empirical data sets and randomly generated parasite distributions in order to assess the optimal sample size as a balance between suitable estimates of the mean abundance and acceptable level of uncertainty in these estimates. Biases and *CI*s were used as measures of accuracy for the simulation modelling. The objective of the present paper is threefold: i) to estimate optimal sample size for parasitological surveys of *Ligophorus* spp.; ii) to evaluate the precision level and *CI*s in samples with different elements, and iii) to test the reliability of each approach to determine the optimal sample size for parasitological studies.

Materials and Methods

Study area, fish sampling and parasite collection

This study is based on 205 dissected individuals *M. cephalus* from three localities, the Kerch Strait, the Sivash Lake and the Balaklava Bay, in the Azov-Black Seas in the period of 2001 – 2013 (Sarabeev, 2015) and one extra sample with 19 fish individuals from the Sivash Lake collected in 2014. The sample sizes ranged between 15 and 35 specimens. Only two-years old, and older fish within the size range of 24 – 65 cm (total length) were used in the analyses. Nine samples were studied across all localities, years and seasons. Collected fish were measured and surveyed for parasites within the day of capture or after freezing. Gills were carefully examined under a stereomicroscope for ectoparasites. All monogeneans were identified and counted. Taxonomic identification was attempted to the species level. Identification of *Ligophorus* spp. followed Sarabeev *et al.* (2013). The present study considers two species of *Ligophorus* from *M. cephalus* across the Azov-Black Sea localities, *L. cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa & Euzet, 2006 and *L. mediterraneus* Sarabeev, Balbuena & Euzet, 2005. For each parasite species, samples with more than 6 infected hosts were considered to avoid inadequate estimation of mean abundance due to very low prevalence (Poulin, 2013). Therefore, the data sets included 197 and 192 fish individuals of which 132 and 96 were infected by *L. cephali* and *L. mediterraneus*, respectively.

Data analysis

The mean abundance was calculated according to Bush *et al*. (1997). The distribution pattern of parasite data was characterized by two parameters, using values of parameter *k* of the NBD and parameter *b* of the Taylor's power law (Taylor, 1961) $s^2 = am^b$ in which s^2 is the sample variance, *m* is the sample mean and *a* is a scaling factor related with the sample size. The dispersion parameter *k* was estimated by using the maximum likelihood method (Bliss & Fisher, 1953; Davis, 1994; Young & Young, 1998). The chi-square statistic (Bliss & Fisher, 1953) was used to test goodness-of-fit of the NBD for empirical data.

Through use of a ln transformation the coefficient *a* and the exponent *b* are estimated by the *y*-intercept antilog and the slope, respectively, of the least square regression line of $\ln s^2$ on $\ln m$ as:

$$\ln s^2 = \ln a + b \ln m \,, \qquad (1)$$

using empirical sample means and variances. The values of *a* and *b* were tested for departure from 0 and 1, respectively, by using a two tailed *t*-test (Snedecor & Cochran, 1980). The coefficients of determination R^2 were calculated, to characterize the fit of Taylor's model. For each parasite species 10 mean-variance pairs (9 samples and one aggregated data set) were obtained from empirical samples.

The analytic approach to determine an optimum sample size (*n*) for mean parasite abundance estimation is based on the general formula (Karandinos, 1976):

$$n = \left(\frac{Z_{\alpha/2}}{D}\right)^2 \frac{s^2}{m^2} \,, \qquad (2)$$

in which $Z_{\alpha/2}$ is the standard normal deviate such that $P(Z > Z_{\alpha/2}) = \alpha/2$; *D* is a level of precision and is used to define half-width of the *CI* as a fixed proportion of the mean ($CI/2 = Dm$ (Wilson, 1985). For a 95 % *CI*, $\alpha = 0.05$, then $Z_{\alpha/2}$ equals 1.96.

In the present study the optimum sample size was determined for two precision levels: $D = 0.5$ and $D = 0.8$. These levels are reasonable for practical applications, and are acceptable in most sampling research (Cyr *et al*., 1992; Mouillot *et al*., 1999, Opit *et al*., 2009).

If the dispersion pattern of the target population is well described by the NBD, Karandinos' equation (2) can be rewritten as:

$$n = \left(\frac{Z_{\alpha/2}}{D}\right)^2 \left(\frac{1}{m} + \frac{1}{k}\right) \,. \qquad (3)$$

Incorporating Taylor's power law into Karandinos' equation (2) the sample size model becomes:

$$n = \left(\frac{Z_{\alpha/2}}{D}\right)^2 am^{b-2} \,. \qquad (4)$$

The BLB using the R statistical data analysis software (version 3.3.3, R Development Core Team, 2017) was applied here in a simulation study to determine the optimal sample size. The effect of sample size on *CI* width was tested for: i) two empirical data sets of *L. cephali* and *L. mediterraneus*; and ii) five simulated data sets with fixed mean and variable *k*. The random 1000-dimension samples with the NBD were generated, parameterized by the fixed value of mean abundance equal to 5.55 and exponent *k* of the NBD ranging between 0.1 and 0.9. This range covers most variation of *k* values found for *Ligophorus* spp. (our unpublished data). The random simulation procedure was implemented using the R function *rnegbin()* in the MASS package (Ripley *et al*., 2017). To examine the effect of sample size on mean abundance, a bootstrapping method was applied to generate 95 % *CI*s for given parasite data set: *n* elements from each data set were randomly selected 10000 times, and then we performed a bootstrap with 5000 iterations and computed the mean for each *n*-dimension sample occasion, based on samples from 10 to 100 elements in steps of 5. The 95 % *CI*s for bootstrap were defined using the values that mark the upper and lower 2.5 % of the bootstrap distribution. Bias significance was evaluated through *t*-test. The difference between the estimates of the mean abundance obtained based on different sample sizes was examined by Dunnett's Modified Tukey-Kramer Pairwise Multiple Comparison Test (DTK) from package "DTK" (Lau, 2015) after a logarithmic transformation of the data. The DTK test allows to conduct a pairwise multiple comparison test for mean differences with no assumption of equal population variances. A significance level of 0.05 was used for all test procedures.

According to the purpose of the study, several criteria were used to determine the appropriate sample size: the fit of empirical data to the theoretical distribution; the desired precision (*CI*s width for mean abundance); the achievement of minimal bias and the comparison of mean abundance differences based on different sample sizes. All criteria had to be met in order to accept a given *n* as the minimum sample size needed for estimation of the parasite mean abundance.

Table 1. Summary data for samples of *Mugil cephalus* surveyed from the Azov-Black Seas with information on abundance, variance and aggregation indices of two helminth species (ln *a*: *y*-intercept, *b*: slope, *SE*: standard error, *t*: *t*-test result, R^2: coefficient of determination, *k*: negative binomial parameter, χ^2: chi-square statistic).

	Mean abundance	Variance	ln *a* (SE, *t*-value)	*b* (SE, *t*-value)	R^2	*k* (SE, χ^2)
Ligophorus cephali	15.65	1908.92	-0.16 (1.11, -0.14*)	2.64 (0.43, 6.1)	0.84	0.25 (0.03, 26.02)
L. mediterraneus	5.55	221.25	0.98 (0.67, 1.47*)	2.33 (0.42, 5.59)	0.82	0.21 (0.03, 2.48**)

*No significantly differs from 0 ($P>0.05$). **The NBD model fits to data ($P>0.05$)

Results

The dispersion pattern of both helminth species could be characterized as a highly aggregated distribution with the variance being substantially larger than the mean values. Obtained values of *k* were lower than 1, also indicating on a highly aggregated distribution of these species in the host (Table 1). The chi-square test revealed that *L. cephali* data set does not fit to the NBD, thus not allowing to determinate the optimal sample size using parameter *k* of the NBD in formula (3). For both parasite species, the ordinary least square regression showed a very strong relationship between the means and variances (R^2 =0.82 and 0.84, P<0.0001) with values of b>2 that also indicates a high degree of aggregation. Because the slope b exceeded 2, formula (4) based on Taylor's power law could not be used (Shelton & Trumble, 1991). For *L. mediterraneus*, minimum sample sizes needed to reach the predetermined precisions D=0.5 and D=0.8 based on formula (3) are 76 and 30, respectively.

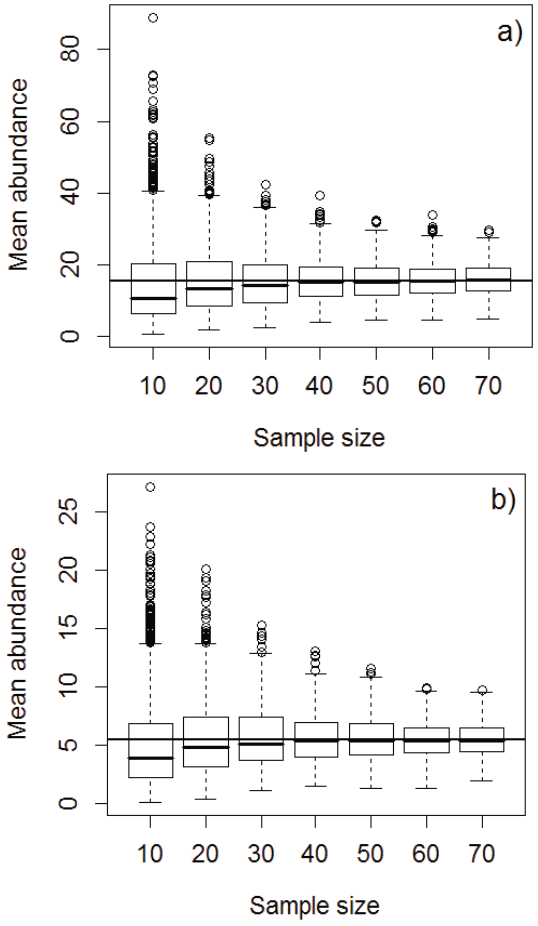

Fig. 1. Distribution of mean abundance obtained from empirical parasite data sets by BLB for different sample sizes for *Ligophorus cephali* (a) and *L. mediterraneus* (b). The box spans the first and third quartiles; the median is marked inside the box by thick horizontal line; minimum and maximum values excluding outliers (whiskers) and outliers (circles); the straight line is the empirical mean abundance.

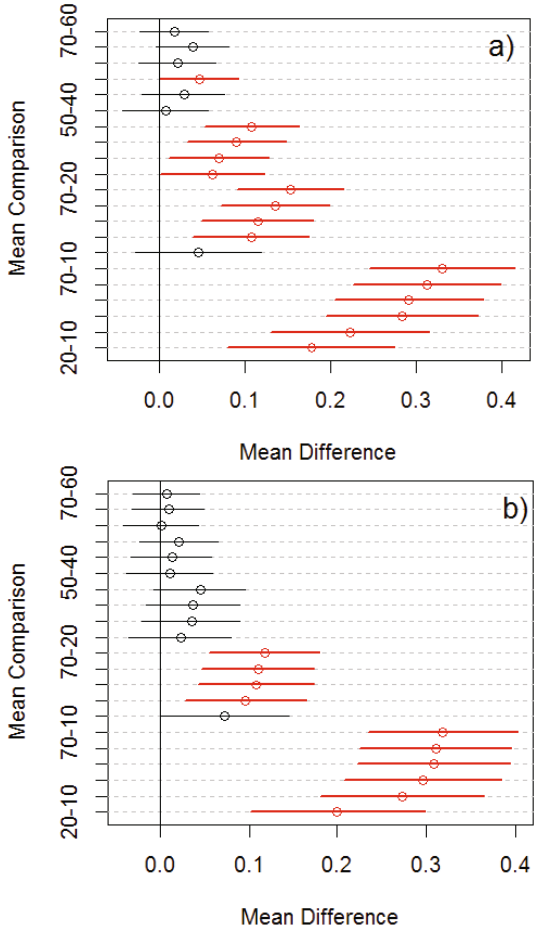

Fig. 2. Simultaneous confidence intervals for all pairwise comparisons of group means. Intervals were computed by the Dunnett's Modified Tukey-Kramer Pairwise Multiple Comparison Test for the mean abundance data of *Ligophorus cephali* (a) and *L. mediterraneus* (b) across different sample sizes. If the interval does not include a zero, the corresponding means are significantly different.

For both parasite species, the mean abundance values obtained by the BLB were close to empirical values of the mean, and no significant biases were found in the estimates. The distribution of mean abundance estimates obtained by simulations was highly right-skewed and the median values were always under-estimating the empirical value at low sample size (Fig.1). The results showed overlapping between the medians of bootstrapping means and the empirical mean abundance beginning from the sample with 40 elements for both examined species. The pairwise statistical comparison between mean abundance values across sample sizes using the 95 % *CI*s is represented in Figure 2. Following the TDK test the estimates related to sample sizes up to 30 and 20 specimens were convincingly different from all others, while there were moderate differences between samples over 40 and 30 fish specimens for *L. cephali* and *L. mediterraneus*, respectively.

The bootstraped 95 % *CI*s were non-symmetric, which correspond to the asymmetry of the underlying mean parasite abundance distributions, and became narrower as sample size increased for both

parasite species (Fig. 3). The effect of sample size on *CI* width as a fixed proportion of the mean for variable values of *k* is shown in Figure 4. The simulation results revealed that as *k* decreases *CI* width was more strongly affected by sample size. The width of the 95 % *CI* is not markedly narrowed with increases in sample size for samples above 25 for *k*=0.9, 30 for *k*=0.5, 40 for *k*=0.2, 45 for *k*=0.15 and 50 for *k*=0.1 elements. For empirical data sets of both species studied here, the largest decrease in *CI* width (exponential phase) was found for sample sizes below 40 individuals, while the further increase of samples resulted in a slow linear decrease in *CI* width (linear phase). This means that the *CI* markedly decreases with increasing sample size up to ca. 70 – 80 specimens. However, further increase of sample size did not really narrow the *CI*. The width of the 95 % *CI*s was decreased from values (1.6 x mean) for sample size with 35 elements to (1 x mean) for sample size with 70 elements in both model species. For *L. cephali* and *L. mediterraneus*, which have close values of the parameter *k* (0.25 versus 0.21) and different values of the mean abundance (15.65 versus 5.55), the variation of the *CI* width was either small or negligible.

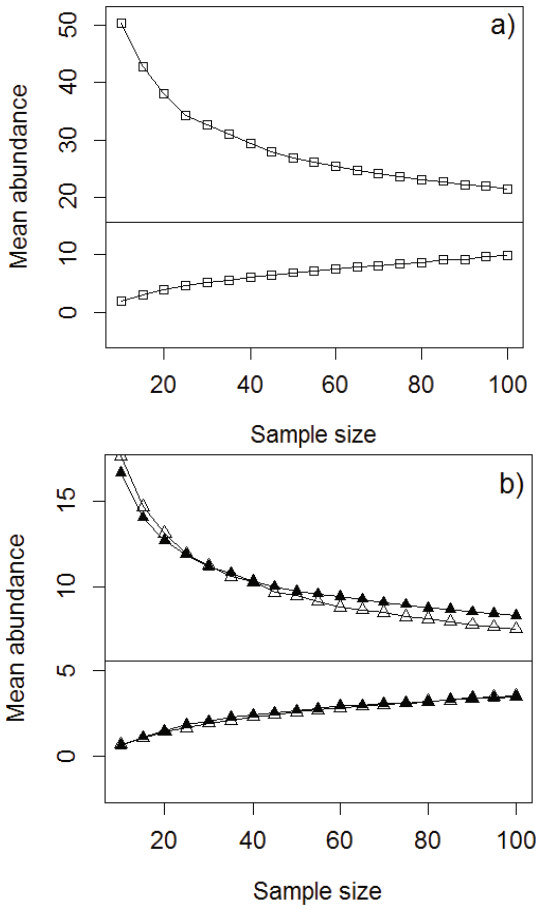

Fig. 3. Mean abundance and its 95 % *CI*s calculated by BLB for different sample sizes for *Ligophorus cephali* (a) and *L. mediterraneus* (b); bootstrap *CI*s based on empirical data set for *L. cephali* (open square); bootstrap *CI*s based on randomly generated and empirical data sets for *L. mediterraneus* (filled and open triangle, respectively); the straight line is the empirical mean abundance.

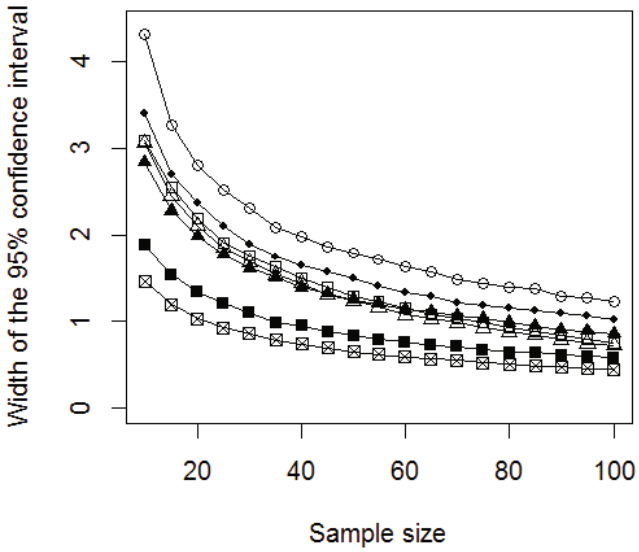

Fig. 4. Width of the 95% CIs as a fixed proportion of the mean abundance determined by BLB from the empirical parasite data sets for *Ligophorus mediterraneus* and *L. cephali* (open triangle and open square, respectively) and randomly generated data with fixed mean abundance of 5.55 and variable *k* of 0.1, 0.15, 0.2, 0.5 and 0.9 (open point, filled point, filled triangle, filled square and square cross, respectively).

Discussion

In the present study, the BLB analysis showed that the minimum required sample size depends greatly on the actual aggregation of the parasite population. The higher degree of variability in the size of parasite infrapopulation, the larger sample size needs to be examined in order to obtain the true value of the mean abundance (Wilson *et al*., 2002). On the other hand, the measure of aggregation will tend to underestimate true aggregation in small samples. This is because heavily infected hosts are rarely found in wild populations and therefore, most likely the probability to be observed in small sample sizes is low (Poulin, 2013). Similarly, the mean abundance calculated from low sample sizes will be underestimated if we do not account for the distribution tail (Marques & Cabral, 2007).

The mean abundance estimates should be reported along with *CI*s, which will allow researchers to assess the biological significance of presented findings (Steidl *et al*., 1997). From a practical point of view, the level of precision is the dominant factor in determining the sample size. Following Buntin (1994), one of the ways to determine the precision is to express it as a confidence interval such that the estimate of the mean should be within a certain value of the true mean with a given probability. Most investigators prefer narrow *CI*s that require large sample sizes for aggregated populations. It stimulates researchers to look for the balance between the limitations of the time and effort required for sample collection, on the one hand, and the essential degree of precision of parasitological indices on the other hand. Because of heterogeneity in parasite infection, it is difficult to apply a theoretical approach for

this purpose. Therefore, the simulation bootstrap procedure based on an empirical data set is a much more robust tool. For small samples, the 95 % bootstrap *CI*s for estimates of the mean abundance are typically very large and skewed upwards. The exponential decrease in the *CI* width as sample size increases indicates the rapidly decrease in the level of uncertainty, and in this way, sample size becomes reasonable for estimation of mean abundance. The further slow linear decreases in the *CI* with sample size increases could be explained by a high total number of non-zero values in such samples. In the example of *L. mediterraneus* and *L. cephali* it was shown that the *CI* becomes more precise and less skewed upwards when sample size is between 35 and 70 fish individuals. Possibly for the reason that such samples are less variable in a number of parasite individuals per host.

Depending on the study aims, researchers may seek higher confidence with a wider interval. For *Ligophorus* species, the reasonable precision level could be chosen between 0.8 and 0.5. If the purpose is to get a general idea about the population abundance, the sample size required could be reduced by lowering the level of precision to *D*=0.8 (Opit *et al*., 2009). If this lower level of precision is used, the sample size with 35 fish specimens is recommended for the estimation of mean abundance for both studied here *Ligophorus* spp. However, for highly aggregated populations, the sample size needs to be sufficiently large to provide a statistically acceptable data for estimation of less abundant parasites (Fig. 4). According to Rózsa *et al*. (2000), the mean abundance is strongly dependent on a few heavily infected individuals; therefore, more specimens may be needed to improve the *CI*s.

Obtained minimum sample size did not substantially depended on the mean abundance of the studied monogeneans, although the difference was about three times fold. The similarity in the sample size required for studied model species could be related to their congeneric relationships that could have the effect on the parasite dispersion pattern. The distributions of both examined species are characterized as a highly aggregated with close values of parameters *k* and *b*. Metazoan gill parasites of fish form non-saturated, multispecies and rich infracommunities in which aggregation ensures cross-fertilization and was found to be an important factor determining the distribution on the gills (Rohde *et al*., 1995; Bagge *et al*., 2005). Monogeneans tend to be more aggregated at lower abundances, what happen because more aggregation is needed as the distance to a potential mate increases with decreasing number of conspecifics (Bagge *et al*., 2005). Our results for the required minimum sample size are in accordance with those of Marques and Cabral (2007) obtained for a system of flatfishes and their parasites.

The values of sample size obtained by the analytical formula show a good correlation with estimates based on the simulation technique. However, the *CI*s based on normal theory are less accurate for skewed distributions, in particular for cases where sample sizes are small (Rózsa *et al*., 2000). Therefore, by utilization of the BLB method a more precise bootstrap *CI*s can be obtained.

The results from this study allowed a direct comparison of sample size estimation by two approaches, analytic and simulation. The advantage of using formulae is the possibility to analyze effects of the precision level, mean abundance, parameter *k* of NBD and parameters of Taylor's model on sample size. The most apparent weakness of the analytic approach is the requirement of the fit of sample data to the theoretical distribution. The application of the formula (3) with parameter *k* of the NBD requires that the *k* value was estimated accurately. For highly aggregated parasite populations, according to the formula (3), the sample size strongly depends on the dispersion of value *k* and the precision of *D*, while the mean abundance >1 does not significantly affect the sample size. Although the Taylor's power law has been widely used due to its statistical stability, the formula (4) for sample size calculation based on the Taylor's model is useful only when $b<2$. This is a significant limitation (Shelton & Trumble, 1991) because the aggregation leads to an increase of coefficient *b* to the critical value 2, or in some cases more than 2. Since the analytic approach is often impossible to apply the non-parametric BLB method is preferable for optimum sample size determination. The primary advantage of bootstrapping is that no assumptions are made on the distribution of the initial data set. Researchers need to assume only that the sample data are independent and representative of the population. The accuracy of estimates obtained by bootstrapping depends on the number of observations in the original sample and the number of resamples. Obviously, large samples are likely to be more representative than small samples.

Conclusions

The holistic approach applied here offers a wide range of appropriate methods to sample size computation and to understand the expected precision level for the mean. While the formulae for sample size estimation may not be very meaningful in practice, their value is that they can provide some strategy in sampling plan before a study. Monte Carlo simulations and bootstrap procedures are powerful techniques for sample size determination. In case of small samples, bootstrapping methods are especially useful to compute the descriptive statistics with associated *CI*s. Such approach is reasonable when dealing with critically endangered species for which low sample sizes are often unavoidable. Regarding sample sizes for parasite data sets with a highly aggregated dispersion pattern, sample size equal 80 or more host individuals allows accurate and precise estimation of mean abundance, whereas for the host sample size in range between 25 and 40 individuals, the median estimates showed minimal bias but the sampling distribution skewed to low values. A sample size of 10 host individuals yields to unreliable estimates, particularly for highly aggregated parasite data sets. These findings will help guide prospective design of sampling plan and will aid researchers in understanding the precision level for the estimated mean abundance in parasitological surveys. At the same time, for the studies aimed to com-

pare epidemiological parameters the question about the optimum sample size remains open. Therefore, the next studies should be focused on the investigating the optimum sample size for comparative studies in parasitology and epidemiology.

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Diplodiscus mehrai Pande, 1937 and *D. japonicus* (Yamaguti, 1936): morphology of developmental stages and molecular data

V. V. BESPROZVANNYKH[1], K. V. ROZHKOVAN[1]*, A. V. ERMOLENKO[1], A. V. IZRAILSKAYA[2]

[1]Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS, Vladivostok, 690022, Russia,
*E-mail: *27.tomcat@gmail.com*; [2]Department of Biodiversity and marine bioresoures, Far Eastern Federal University, 690051, Vladivostok, Russia

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Summary

Specimens of the snails *Anisus centrifugops* were infected with two types of cercariae relating to the genus *Diplodiscus,* differing from each other by body and organ sizes were found in a pond in the territory of Vladivostok (Primorsky region, Russia). Further study of their morphology and life cycles allowed us to establish that these flukes belong to *Diplodiscus japonicus* (Yamaguti, 1936) and *D. mehrai* Pande, 1937. Morphological and molecular data confirmed the validity of *D. japonicus* which had previously been synonymized with *D. amphichrus* Tubangui*,* 1933 and *D. mehrai* Pande, 1937.
Keywords: *Diplodiscus mehrai*; *Diplodiscus japonicus*; life cycle; cercariae; metacercariae; adult worm

Introduction

The family Diplodiscidae Cohn, 1904 is a small group of paramphistomoids, found predominantly in amphibians but with representatives in reptiles and in fish (Jones, 2005). The concept of *Diplodiscus* Diesing, 1836, as well as the taxonomy of individual species of this genus, is based on the morphology of adult worms (Sey, 1991). After revision of Amphistomida, and particularly, *Diplodiscus*, most of the species and subspecies from this genus described before 1991 were synonymized to different species (Sey, 1991). At present, there is a small amount of data on developmental stages of *Diplodiscus*. For *D. subclavatus* (Goeze, 1882), *D. amphichrus* Tubangui*,* 1933*, D. brevicoeca* Richard, Chabaud et Brygoo, 1968 (Sey, 1991) and *D. japonicus* (Yamaguti, 1936), only cercariae, metacercariae, and parthenitae have been described (Sey, 1991; Skrjabin, 1949). However, the morphometric data for these stages are incomplete. Besides the life cycles of *Diplodiscus*, data on the circulation features of *D. subclavatus* were obtained. The life span of cercariae after it emergence from a snail (usually in the morning) is approximately 28 h (Grabda-Kazubska, 1980). Cercariae encyst at the end of the free-living phase. Grabda-Kazubska also showed that cercariae encyst in water, but not on amphibian skin as proposed by Lang (1892), and that the life span of metacercariae is 5 days.

The only species of *Diplodiscus* known from Russia is *D. subclavatus*. The literature also reports *Diplodiscus sphincterostoma* at the territory of the Primorsky Region, discovered by E.V. Belous. However, this fluke is not described; thus, this species was considered a “nomen nudum” by Ryzhikov *et al.* (1980).

In a pond in the territory of Vladivostok, we found the snail *Anisus centrifugops* (Prosorova et Starobogatov) with two types of cercariae of *Diplodiscus*. These cercariae differed in body and organs sizes. Further studies of the life cycles, morphology of different life stages of adult worms allowed us to establish that these flukes belonged to *D. japonicus* and *D. mehrai* Pande, 1937.

* – corresponding author

Material and Methods

Morphology and life cycle

A total of 250 specimens of *Anisus centrifugops* naturally infected with parthenitae of species of *Diplodiscus* were taken from a small pond in the territory of Vladivostok, Primorsky Region, Russia. Adult flukes were also found in naturally infected *Rana dybowskii* caught in the same place in the spring and autumn. In order to identify these trematodes and their circulation, four infected snails were separately placed in dishes with 1 l of tap water. The snails were divided according to the size of cercariae: two reservoirs contained mollusks with smaller cercariae, and two others contained mollusks with larger ones. Fifteen tadpoles of *Rana dybowskii* were then placed in each reservoir. Tadpoles were collected in natural ponds free of snails; 30 tadpoles were checked to verify absence of infection before the experiment. During the experiment, tadpoles were periodically dissected to check the level of infection and time of development to the adult worm stage. The process of trematode invasion into the definitive host was studied by placing infected snails into deep Petri dishes. After the peak of cercariae emission, the snail in each dish was replaced with three tadpoles. The process of invasion was observed under a stereo microscope. To check the infection in tadpoles, they were dissected on the first and the subsequent two days from the beginning of the experiment.

To determine the life span of cercariae, infected snails were placed in Petri dishes with tap water for 20 – 30 min. Cercariae emerging within this time were counted as even-aged. Maximal cercariae life span was defined by the time of emergence from the mollusk until the death of all specimens. Phototaxy was tested by placing the cercariae into long, narrow glass vials (20 × 2 × 3 cm), 1/3 of which were covered by black paper from one side, and a bright spotlight placed on the other side. The diurnal rhythm of cercariae emission was observed by placing a single snail in a Petri dish with 50 ml of tap water. Dishes were shaken every 2 h, and 10 aliquots of 1 ml were taken from each dish and placed in smaller vessels. Dishes were then refilled with water to 50 ml. Cercariae were immobilized by iodine and their number in each vessel was counted. The average number of larvae was then multiplied by 5. Experimental studies were conducted at water temperatures ranging between 18 and 22 °C.

All measurements, in millimeters (mm), of metacercariae and parthenitae were made on living specimens, and before measurements, cercariae were fixed in hot 4 % formalin. Adult worms were fixed in 70 % ethanol and then placed in 96 % ethanol. For whole mounts, worms were stained in alcoholic hydrochloric carmine, dehydrated in a graded ethanol series, cleared in clove oil, and mounted in Canada balsam.

Extraction of total genomic DNA, PCR, and sequencing

Two and four adult specimens of *D. mehrai* and *D. japonicus*, respectively, were washed several times in a physiological solution immediately after dissection and stored in 96 % ethanol. Genomic DNA was extracted from individual worms using hot sodium hydroxide and Tris (HotSHOT) technique (Truett *et al.*, 2000). The complete sequences of 18S rDNA, ITS1-5.8S-ITS2 rDNA region and partial sequences of 28S rDNA were amplified using the following primer sets: 18S-E and 18S-F (Littlewood & Olson, 2001) for 18S rDNA, BD1 and BD2 (Morgan & Blair, 1995) for ITS1-5.8S-ITS2 rDNA, and digl2 and 1500R (Tkach *et al.*, 2003) for 28S rDNA. PCR reaction was conducted in a 25 µl reaction mixture containing 10 pM of each primer, 12.5 µl of PCR Master Mix (2X) (Thermo Fisher Scientific Baltics), and 5 µl of extracted DNA. Sequencing was performed on an ABI 3130 automated capillary sequencer (Life Technologies, Grand Island, New York, USA) using PCR primers for 18S rDNA and the following internal primers: 373C, 892C, 1262, 1262C, 1/F (Krieger *et al.*, 2006), Pace-A, 18S-A27, 18S-4 (Littlewood & Olson, 2001). ITS1-5.8S-ITS2 rDNA region was sequenced using PCR primers and the internal primer 3S (Littlewood & Olson, 2001). 28S rDNA was sequenced with ECD2, 900F and 1200R internal primers (Waeschenbach *et al.*, 2007).

Sequence alignment and phylogenetic analyses

The ribosomal DNA sequences were assembled and aligned manually using the MEGA 5 (Tamura *et al.*, 2011). Sequences have been submitted to GenBank under the following accession numbers: KX506856-KX506857 (*D. mehrai*) and KX506852-KX506855 (*D. japonicus*).

For the purpose of phylogenetic analysis we used the combined set of 18S and 28S rDNA sequences. Prior to phylogenetic analyses sequences were edited, the ends of each sequence were trimmed to match the shortest sequence in the alignment. The final

Table 1. List of taxa, incorporated into the molecular analysis

Species	n	Reference	Accession number
Diplodiscus mehrai	2	Current study	KX506856-KX506857
Diplodiscus japonicus	4	Current study	KX506852-KX506855
Diplodiscus subclavatus	1	Olson *et al.*, 2003	AY222212
Fischoederius cobboldi	3	Ghatani *et al.*, 2014	JX518961-JX518963
Fischoederius elongatus	3	Ghatani *et al.*, 2014	JX518964-JX518966
Fasciola hepatica	1	Olson et al., 2003	AY222244

alignment yielded was 2874 nucleotide long and consisted of 13 sequences (Table 1). Sequences of *Fasciola hepatica* (Linnaeus, 1758) were used as outgroup. Distance matrices were constructed with the absolute pairwise character difference. Pairwise comparisons of absolute sequence divergence for all taxa were calculated with gaps treated as missing data. Maximum Likelihood (ML) using PhyML 3.1 (Guindon & Gascuel, 2003), and Bayesian Inference (BI) using Mr. Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003) analyses of the 18S-28S rDNA data were conducted to explore relationships between the newly generated data and nucleotide sequences available in NCBI Genbank. ML and BI phylogenetic analyses were performed using TIM3+G substitution model with gamma correction value 0.1190. This model showed the best fit to our data using Bayesian information criterion (BIC) in jModeltest 2.1.5 software (Darriba *et al.*, 2012). Bayesian inference was used with the following nucleotide substitution parameters: Iset nucmodel = 4by4, nst = 6, and rates = gamma. Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations, log-likelihood scores were plotted and only the final 75 % of trees were used to produce the consensus tree. This number of generations was considered sufficient because the standard deviation of spitted sequences value dropped below 0.01 at the end of the run. The significance of the phylogenetic relationship was estimated using a bootstrap analysis (Felsenstein, 1985) with 100 replications for ML

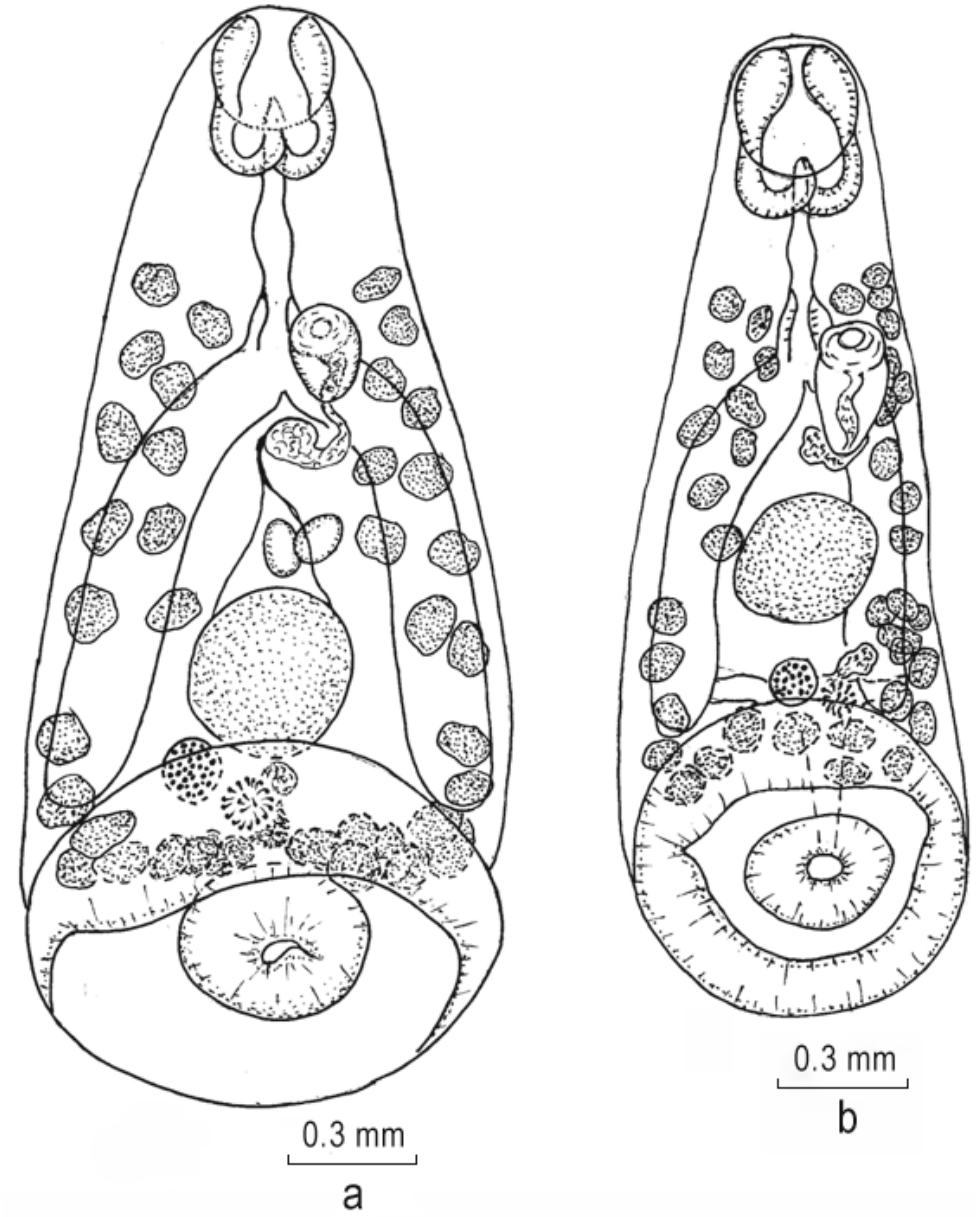

Fig. 1. Adult *Diplodiscus*: a – *D. mehrai* Pande, 1937, b – *D. japonicus* (Yamaguti, 1936).

Table 2. Measurements (mm) of adult *Diplodiscus*.

Feature	*D. japonicus* (present study)	*D. japonicus* (from: Yamaguti 1936)	*D. japonicus* (according to Bravo, 1941; from: Skrjabin 1949)	*D. mehrai* (present study)	*D. mehrai* (according to Pande, 1937; from Skrjabin 1949)	*D. mehrai* (from: Sey, 1991)	*D. amphichrus* (according to Tubangi, 1933; from: Skrjabin 1949)	*D. amphichrus* (from: Sey, 1991)
Body length	1.25 – 2.00 (0.80 – 0.89)	1.5 – 2.5	1.8 – 2.75	2.37 – 2.77 (0.98 – 1.03)*	2.1 – 3.2	2.1 – 3.2	1.60 – 3.45	1.6 – 3.4
Body width	0.57 – 0.99 (0.41 – 0.49)	0.4 – 1.0	0.74 – 0.975	0.91 – 1.23 (0.36 – 0.48)	1.0 – 1.3	1.0 – 1.3	0.60 – 0.86	0.2 – 0.8
Pharynx length	0.20 – 0.31 (0.112 – 0.168)	–	0.156 – 0.292	0.25 – 0.28 (0.123 – 0.168)	0.36	0.36	0.110 – 0.292	0.29 – 0.32
Pharynx width	0.18 – 0.31 (0.145 – 0.168)	0.15 – 0.23	0.195 – 0.214	0.22 – 0.31 (0.134 – 0.145)	0.2 – 0.32	–	0.100 – 0.330	–
Pharyngeal sacs length	0.18 – 0.20 (0.10 – 0.11)	–	0.117	0.17 – 0.24 (0.14 – 0.15)	0.2	0.28	0.136	0.13
Pharyngeal sacs width	0.13 – 0.22 (0.09 – 0.10)	–	0.078 – 0.097	0.19 – 0.22 (0.089 – 0.112)	0.18	–	0.136	–
Pharynx together with sacs length	0.26 – 0.43	0.25 – 0.37		0.32 – 0.43				
Esophagus length	0.18 – 0.39	0.33	0.273 – 0.33	0.28 – 0.50	0.38 – 0.50	0.38 – 0.50	0.270 – 0.440	0.35
Esophageal bulb length	0.120 – 0.154 (0.041 – 0.056)	0.12 – 0.15	0.102 – 0.175	0.123 – 0.169 (0.084)	0.11 – 0.14	0.11 – 0.150	0.156	–
Esophageal bulb width	0.108 – 0.123 (0.041 – 0.056)	0.10 – 0.13	0.085 – 0.117	0.108 – 0.139 (0.056 – 0.072)	0.07 – 0.10	–		–
Acetabulum length	0.45 – 0.65 (0.26 – 0.35)	0.47 – 0.80	0.2	0.62 – 0.95 (0.32 – 0.40)	0.56 – 0.74	0.56 – 0.74	0.50 – 1.08	0.54 – 1.16
Acetabulum width	0.54 – 0.89 (0.39 – 0.59)	0.47 – 0.80	0.7 – 0.975	0.82 – 1.19 (0.40 – 0.54)	0.90 – 1.11	0.90 – 1.14	0.50 – 1.08	0.54 – 1.16
Sucker-like protuberance length	0.17 – 0.31	–	–	0.34 – 0.40	–	–		–
Sucker-like protuberance width	0.20 – 0.35	–	–	0.38 – 0.47	–	–		–
Ovary length	0.11 – 0.13 (0.05 – 0.069)	0.13 – 0.25	0.102 – 0.175	0.15 – 0.17 (0.061 – 0.072)	0.16 – 0.18	0.16 – 0.18	0.12 – 0.24	–
Ovary width	0.12 – 0.17 (0.05 – 0.069)	0.16 – 0.20	0.062 – 0.128	0.15 – 0.17 (0.056 – 0.072)	0.16 – 0.18	0.16 – 0.18	0.12 – 0.24	–
Testis length	0.32 – 0.37 (0.084 – 0.089)	0.18 – 0.37	0.198 – 0.292	0.36 – 0.45 (0.112 – 0.123)	0.23 – 0.40	0.23 – 0.40	0.140 – 0.1487	0.14
Testis width	0.30 – 0.50 (0.112 – 0.123)	0.20 – 0.33	0.253 – 0.312	0.38 – 0.42 (0.100 – 0.170)	0.34 – 0.45	0.34 – 0.45	0.140 – 0.1487	0.14
Cirrus-sac length	0.127 – 0.189 (0.089 – 0.108)	0.16	–	0.21 – 0.23 (0.092 – 0.139)	0.16	0.18	0.12 – 0.22	0.13 – 0.15
Cirrus-sac width	0.060 – 0.135 (0.054 – 0.069)	0.10	–	0.14 – 0.20 (0.058 – 0.081)	0.18	0.16	0.10 – 0.14	0.09 – 0.12
Number of vitelline follicles	17 – 21	30 – 40	21	17 – 21	35 – 40	17 – 20	45	–
Eggs length	0.095 – 0.104	0.102 – 0.126	0.105 – 0.120	0.112 – 0.126	0.09 – 0.14	0.146	0.104 – 0.112	0.113 – 0.119
Eggs width	0.061 – 0.073	0.066 – 0.078	0.058 – 0.097	0.067 – 0.078		0.093	0.062 – 0.070	0.068 – 0.079
Body length/length of pharynx with sacs	4.5 – 4.9	–	–	6.2 – 7.4	–	–	–	–
Acetabulum/pharynx	1.93 – 2.4	–	–	2.48 – 3.39	–	–	–	–
Genital pore	Bifurcal or postbifurcal	Bifurcal	Postbifurcal	Bifurcal or postbifurcal	Bifurcal	Bifurcal	Postbifurcal	Postbifurcal (on some distance – Sey 1991, Fig. 314)

* - Sizes at the time of puberty

and by the amount of a posterior probability for BI. Trees were visualized using FigTree 1.4 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Description

***Diplodiscus mehrai* Pande, 1937**

Definitive host: tadpoles and adult *Rana dybowskii* Günther (Ranidae, Rafinesque).
Infection rate of tadpoles and frogs in natural conditions: 100 %.
Infection intensity of tadpoles in natural conditions: 4 – 23 specimens.
Infection intensity of frogs in natural conditions: 1 – 15 specimens.
Infection rate of tadpoles in experiment: 20 %.
Infection intensity of tadpoles in experiment: 1 – 7 specimens.
Site: rectum.
Intermediate host: freshwater snail *Anisus centrifugops* (Prosorova et Starobogatov) (Planorbidae, Rafinesque).
Infection rate: 4.2 %.
Locality: Vladivostok, Primorsky Region, Russia; 43°12' N, 131°55'E.

Adult worm (based on 10 specimens; Table 2, Figs. 1a, 3a): Body conical, non-spined with narrow anterior end and wide posterior end. Pharynx terminal with two pharyngeal sacs. Specimens from tadpoles with diffuse pigment spots on both sides of these sacs. Specimens from frogs with pigment spots or not. Oesophagus ventral to pharyngeal sacs. Oesophageal bulb with wall thickness 0.027 – 0.031. Oesophageal bifurcation on border of anterior and middle third of body. Caeca reach anterior margin of acetabulum. Acetabulum terminal, on posterior end of body, with central sucker-like protuberance. Testis single, spherical, smooth to irregular, between caeca on median line of body before acetabulum. Cirrus-sac in area of oesophageal bifurcation, with small internal seminal vesicle. External seminal vesicle present, size depends on its occupancy with sexual products. Genital pore bifurcal to postbifurcal, right or left from bifurcation. Ovary dextral from medial line of body on level of posterior margin of testis. Mehlis' gland left from ovary, 0.16 × 0.15. Uterus between caeca, genital pore and testis. Each of two vitelline fields form two groups of follicles, one between level of middle of oesophagus and middle of testis, second between posterior margin of testis and acetabulum. Most of second group of follicles in both vitelline fields across body, and follicles reach median line of body. Each vitelline field comprises 17 – 21 follicles. Eggs not numerous, operculated. Excretory bladder T-shaped.
Redia (based on 10 specimens; Fig. 2a.) Body sac-shaped, 0.10 – 0.13 × 0.28 – 0.3. Pharinx 0.09 – 0.12 × 0.11 – 0.12. Caecum short.
Cercaria (based on 10 specimens; Table 3, Figs. 2b, 3b) Body leaf-shaped, slightly transparent, grey by pigment. Pigment eyes

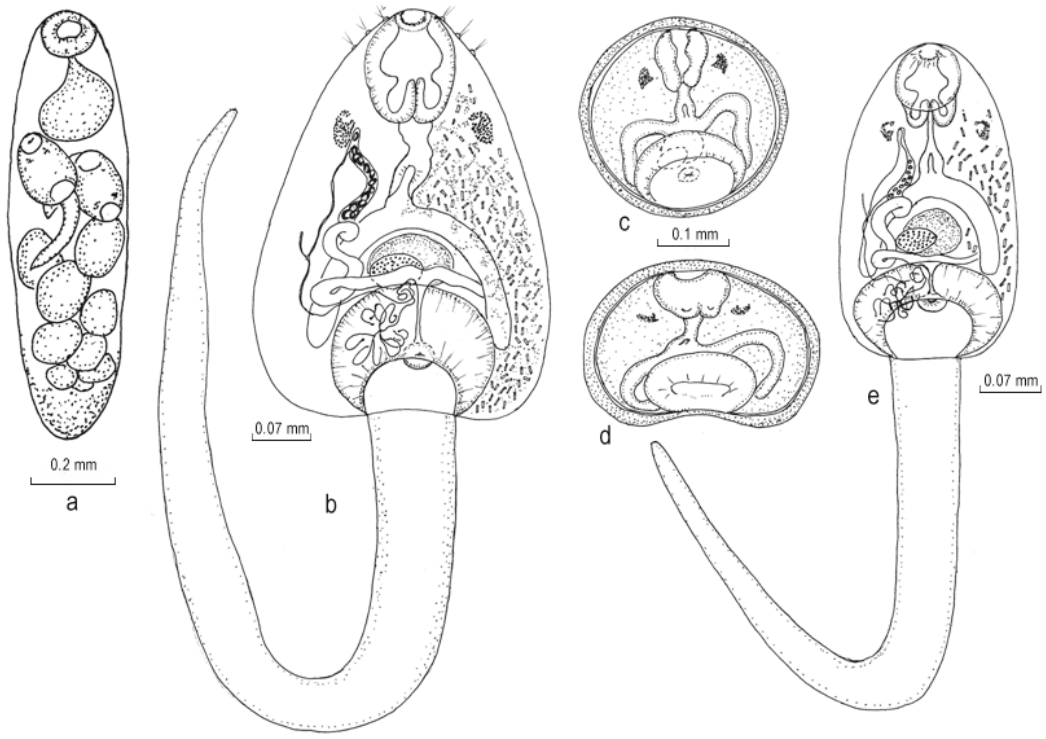

Fig. 2. *Diplodiscus mehrai* Pande, 1937: a – redia, b – cercaria, c and d – metacercaria, dorso-ventrally and laterally, accordingly; *D. japonicus* (Yamaguti, 1936): e – cercaria.

Fig. 3. *Diplodiscus mehrai* Pande, 1937: a – adult worm, b – cercaria; *D. japonicus* (Yamaguti, 1936): c – adult worm, d – cercaria; e – metacercaria found in feces of snail.

on both sides of body. Anterior end of body with long sensillae. Pharynx terminal with two sacs. Oesophagus short, with bulb. Oesophageal bifurcation on border of anterior and middle third of body. Caeca terminate at level of middle of acetabulum. Acetabulum terminal, on posterior end of body, with central sucker-like protuberance. Primordium of testis cross-oval, immediately before acetabulum. Primordium of ovary left from median line of body, on level of testis. Cystogenous glands from pharyngeal sacs to posterior end of body. Excretory bladder T-shaped. Two main collective channels, each with 20 – 21 granules, left and right from median line of body, turn in area of pigment eyes to posterior end of body. On midline of body each channel divided on two secondary channels; one of them reaches pharynx and second reaches acetabulum, and further divided into three branches, each includes three ducts terminated by small expansion. Tail 1.7 – 2.1 times longer than body.

Metacercaria (based on 10 specimens; Fig. 2c,d). Cyst 0.28 × 0.28 – 0.29, spherical, concaved on one side.

Molecular sequence data 18S rDNA (complete) – 1991 bp, ITS1 (complete) – 490 bp, 5.8S rDNA (complete) – 159 bp, ITS2 (complete) – 281 bp, 28S rDNA (partial) – 1391 bp.

***Diplodiscus japonicus* (Yamaguti, 1936)**

Definitive host: tadpoles and adult *Rana dybowskii* Günther (Ranidae, Rafinesque).

Infection rate of tadpoles and frogs in natural conditions: 100 %.

Infection intensity of tadpoles in natural conditions: 6 – 32 specimens.

Infection intensity of frogs in natural conditions: 1 – 21 specimens.

Infection rate of tadpoles in experiment: 43 %.

Infection intensity of tadpoles in experiment: 1 – 95 specimens

Site: rectum.

Intermediate host: freshwater snail *Anisus centrifugops* (Prosorova et Starobogatov) (Planorbidae, Rafinesque).

Rate of infection: 6.4 %.

Locality: Vladivostok, Primorsky Region, Russia; 43° 12'N, 131° 55'E.

Adult worm (based on 10 specimens; Table 2, Figs. 1a, 3c). Body conical or trapezoidal, non spined with narrow anterior end and wide posterior end. Pharynx terminal with two pharyngeal sacs. Specimens from tadpoles with diffuse pigment spots on both sides of these sacs. Specimens from frogs with pigment spots or not. Oesophagus ventral to pharyngeal sacs. Oesophageal bulb oval, with wall thickness 0.039 – 0.042. Oesophageal bifurcation on border of anterior and middle third of body. Caeca reach anterior margin of acetabulum. Acetabulum terminal, on posterior end of body, with central sucker-like protuberance, can partly cover ovary. Testis spherical, smooth to irregular, between caeca on median line of body before acetabulum. Cirrus-sac in area of oesophageal

bifurcation, with small internal seminal vesicle. External seminal vesicle present. Genital pore bifurcal to postbifurcal. Ovary between testis and acetabulum, on median line of body, on some distance from testis. Mehlis' gland left from ovary, 0.11 × 0.12. Uterus between caeca, genital pore and ovary. Each of two vitelline fields rorm two groups of follicles. One between level of middle of oesophagus and middle of testis, second between posterior margin of testis and acetabulum Most of second group of follicles in both vitelline fields across body, and follicles reach median line of body. Each vitelline field comprises 17 – 21 follicles. Eggs not numerous, operculated. Excretory bladder T-shaped.

Redia (based on 10 specimens). Body sac-shaped, 0.10 – 0.13 × 0.28 – 0.3. Pharinx 0.09 – 0.12 × 0.11 – 0.12. Caecum short.

Cercaria (based on 10 specimens; Table 3, Figs. 2e, 3d). Body leaf-shaped, transparent. Pigment eyes on both sides of body. Anterior end of body with poorly visible, short sensillae. Pharynx terminal with two sacs. Oesophagus short, with bulb. Oesophageal bifurcation on border of anterior and middle third of body. Caeca terminate at level of anterior margin of acetabulum. Acetabulum terminal, on posterior end of body, with central sucker-like protuberance. Primordium of testis cross-oval, immediately before acetabulum. Primordium of ovary right from median line of body, on level of testis. Cystogenous glands from pharyngeal sacs to posterior end of body. Excretory bladder T-shaped. Two main collective channels, each with 21 – 23 granules, left and right from median line of body, turn to posterior end of body. On midline of body each channel divided on two secondary channels; one of them reaches pharynx and second reaches acetabulum, and further divided into three branches, each includes three ducts terminated by small expansions. Tail 2.3 – 2.4 times longer than body.

Metacercaria (based on 10 specimens). Cyst 0.23 – 0.24 × 0.25 – 0.26, spherical, concaved on one side.

Molecular sequence data 18S rDNA (complete) – 1991 bp, ITS1 (complete) – 469 bp, 5.8S rDNA (complete) – 159 bp, ITS2 (complete) – 281 bp, 28S rDNA (partial) – 1391 bp.

Life cycles

For both studied species of *Diplodiscus,* as for other representatives of this genus, the two-host life cycle is usual. Cercariae (1 – 50 specimens) emerged from snails between 11 a.m. and 2 p.m. The most intensive emergences, up to 50 specimens per day, of cercariae occurred during spring. In August – September, this value decreased to 1 – 10 cercariae per day. Larvae are active in water and have positive phototaxis. This allows them to concentrate in warm and sunny pond regions. Such places contain many tadpoles. These factors presumably improve the possibility of definitive host infection. The life span of the free-living form in water temperature 18 – 22 °C is 8 – 12 h. At the end of this time, some cercariae are encysted. The rest, up to 80 %, do not encyst and die. Metacercariae did not attach to tadpole skin nor to the bottom of the vessel nor to plants and sand in the vessel, and their life span under laboratory conditions was no longer than 3 – 4 days. Larvae can frequently be swallowed by snails but are not digested and stay alive. Such metacercariae were found in snail feces (Fig. 3e).

The results of experiments reported here showed that tadpoles infection occurred mainly by swallowing the cercariae. Tadpoles, after being placed with cercariae, swallowed all of them within 20 – 30 min. At this moment, cercariae were not yet encysted and metacercariae were absent. Tadpoles were dissected over 3 days

Table 3. Sizes (mm) of the cercariae and metacercariae of *Diplodiscus*.

Features	*D. mechrai* (present study)	*D. japonicus* (present study)	*D. japonicus* (from: Yamaguti 1940)	*D. amphichrus* (according to Tubangi & Masilungan; from: Sey 1991)	*D. amphichrus* (according to Odening; from: Sey 1991)
			Cercaria		
Body length	0.51 – 0.67	0.30 – 0.36	0.36 – 0.75	0.21 – 0.24	0.41 – 0.59
Body width	0.30 – 0.37	0.19 – 0.26	0.16 – 0.35	0.20 – 0.22	0.27 – 0.29
Pharynx with sacs length	0.12 – 0.14	0.084 – 0.127	0.080 – 0.120	–	0.12
Pharynx width	0.089 – 0.095	0.061 – 0.072	0.054 – 0.084	–	0.09 – 0.15
Pharyngeal bulb length	0.038 – 0.045	0.033 – 0.035	0.027 – 0.045	–	–
Pharyngeal bulb width	0.028 – 0.034	0.014 – 0.019	0.020 – 0.033	–	–
Acetabulum length	0.12 – 0.15	0.112 – 0.140	0.096 – 0.165	–	0.15 – 0.18
Acetabulum width	0.22 – 0.30	0.150 – 0.178	0.130 – 0.204	–	0.15 – 0.18
Primordium of ovary length	0.035 – 0.036	0.028 – 0.034	–	–	–
Primordium of ovary width	0.056 – 0.058	0.045 – 0.056	–	–	–
Primordium of testis length	0.059 – 0.062	0.055 – 0.058	0.030	–	–
Primordium of testis width	0.089 – 0.091	0.061 – 0.063	0.060	–	–
Tail length	1.12 – 1.16	0.72 – 0.83	0.60 – 0.90	0.76 – 1.00	0.56 – 0.69
Tail widht	0.11 – 0.13	0.078 – 0.089	0.075 – 0.130	–	–
			Metacercaria		
Cyst	0.28 – 0.29 in diameter	0.23 – 0.24 × 0.25 – 0.26	0.27 × 0.25	0.24 – 0.25 × 0.20 – 0.23	–

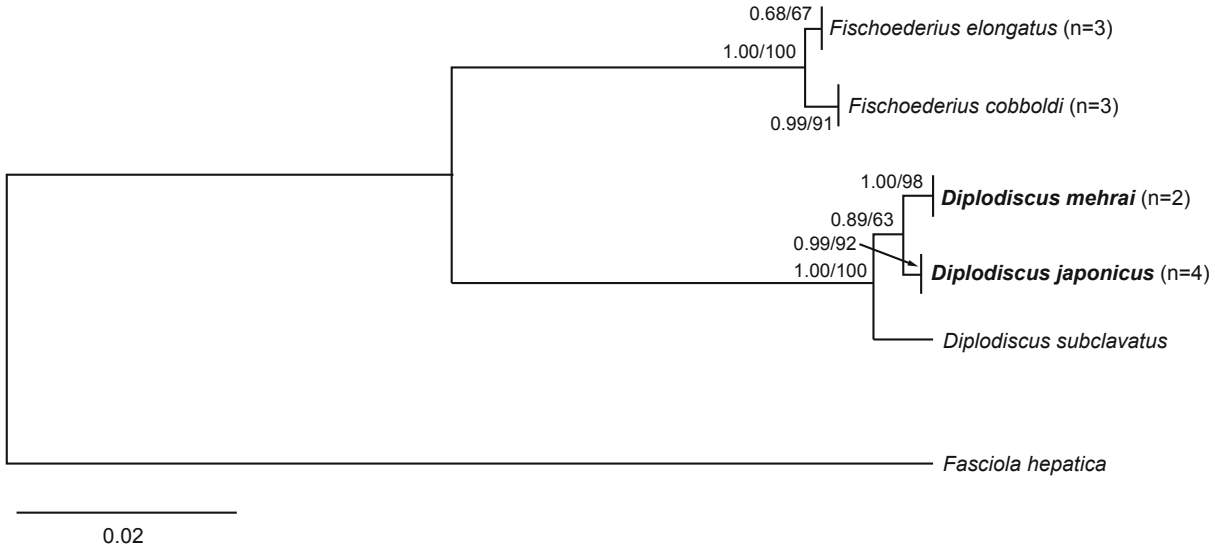

Fig. 4. Bayesian tree inferred from combined 18S and 28S rDNA sequence data. Results of statistical support are shown as follows: BI/ML. Sequences obtained in this study are in bold.

post-exposure, and 7 – 20 flukes were found in their intestines. In other cases, cercariae and metacercariae were found in the tadpoles' oesophagus. Thus, tadpole infection can occur via swallowing either cercariae or metacercariae. However, because only some cercariae are encysted, and the life span of metacercariae is up to 4 days, the primary way of infection occurs through the swallowing of cercariae. Metacercariae can only increase the probability of infection for a short period of time. The first adult *D. mehrai* and *D. japonicus* were both recovered on 30th day of the experiment. *Diplodiscus mehrai* infected 6 tadpoles with an intensity of 1 to 7 flukes, and *D. japonicus* infected 13 tadpoles with an intensity of 1 to 95 flukes.

According to the natural conditions of the Primorsky Region and biological features of *Rana dybowskii*, the maximal number of tadpoles in ponds is observed in May – June, and the minimal one is in August – September (Kuzmin, 2012). These periods correspond to maximal and minimal emission of *Diplodiscus* cercariae, respectively. The tadpoles collected in May – June were infected by both adult flukes and larvae, whereas adult frogs studied in September were infected by adult worms only. This allows us to propose that frog infection in nature occurs at the tadpole stage, and not at adult *Rana*.

Molecular data

The 18S-ITS1-5.8S-ITS2-28S rDNA sequence length for *D. mehrai* was 2412 bp, and 2391 bp for *D. japonicus*. No intraindividual variation was found in any rDNA region examined. For genetic distances calculation and phylogenetic reconstructions we chose the 18S and 28S rDNA genes as the most prevalent in Genbank for the selected group. The main differences between *D. mehrai* and *D. japonicus* were in one G↔A transition for 18S rDNA and 8 transitions (5 G↔A and 3 C↔T) and one G↔T transversion for 28S rDNA. The genetic divergence between *D. mehrai* and *D. japonicus* was 0.35 %, which is equal to that between *Fischoederius cobboldi* and *F. elongatus*. The differences relative to other representative of *Diplodiscus*, *D. subclavatus*, were 0.87 and 0.8 % for *D. mehrai* and *D. japonicus*, respectively. Phylogenetic analysis (Fig. 4) showed that *D. mehrai* and *D. japonicus* are separate species and they are more closely related to each other than to *D. subclavatus*.

Discussion

Sey (1991) revised all species belonging to superfamily Paramphistomoidea Fischoeder, 1901 and included 11 species in *Diplodiscus*. As a result, specimens that had previously been attributed to a single species were synonymized to different species within *Diplodiscus* (Sey, 1991). In part, this was related to *D. mehrai* and *D. amphichrus japonicus* Yamaguti, 1936. From these two species, only *D. mehrai*, described by Pande in 1937 was included in the 11 valid species within *Diplodiscus* (Sey, 1991). Specimens found later and identified as *D. mehrai* were synonymized to *D. magnus* (Srivastava, 1934) (Sey, 1991). Li (1937) *D. amphichrus japonicus* was elevated to species rank and its name was changed to *D. japonicus*. Later, specimens described as *D. amphichrus japonicus* and *D. japonicus* were synonymized either to *D. amphichrus* or *D. mehrai* (Sey, 1991). All flukes listed above were discovered in East-Asia and India. *D. mehrai* was also found in Georgia (Petriashvili, 1964).

The results of our research demonstrate the presence of two species of *Diplodiscus* in the territory of the Primorsky Region. Adult worms of one of these species by metric and morphological characteristics are identical to *D. mehrai* described by Pande (1937) (Table 2). The second species has much in common morphologi-

cally with *D. japonicus* described by Yamaguti (1936, 1941) (Tables 2, 3). The only significant difference between *D. japonicus* from this study and that of Yamaguti is maximal cercariae body size (Yamaguti, 1940). According to Yamaguti the maximal body size is twice the minimal one. Such differences in size of cercariae are not typical for trematodes from one species. All other morphological traits of cercariae, metacercariae, and adult worms and the participation of snails from *Anusis* in the circulation of flukes, points to that the second trematode discovered in Primorsky Region, in our opinion, is *D. japonicus*. This species differs from both adult worms and cercariae of *D. mehrai* and *D. amphichrus* by morphometric features. *Diplodiscus japonicus* differs from adult *D. mehrai* by smaller maximal body size, length of oesophagus, width of ventral sucker and cirrus sac, and maximal egg length. Adult specimens of *D. japonicus* differ from *D. amphichrus* by maximal body size and ventral sucker, testis size, length of pharyngeal sac and position of genital pore (Table 2). Cercariae of *D. japonicus* also have sufficient metric differences from those from *D. amphichrus* and *D. ehrai* (Table 3).

Thus, in most morphometric characters of the developmental stages, *D. japonicus* differs from both *D. mehrai* and *D. amphichrus* (Tables 2, 3), which allows us to count *D. japonicus* as a separate species within *Diplodiscus*. The validity of *D. mehrai* and *D. japonicus* is also confirmed by molecular data. Genetic *p*-distances and phylogenetic analysis (Fig. 4) showed that *D. mehrai* and *D. japonicus* are separate species and that they are more closely related to each other than to *D. subclavatus*.

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Morphology of *Emoleptalea nwanedi* n. sp. from *Schilbe intermedius* from Nwanedi-Luphephe Dam, Limpopo Province, South Africa

P. H. KING[1], W. J. SMIT[2], C. BAKER[3], W. J. LUUS-POWELL[2]*

[1]Department of Biology, Sefako Makgatho Health Sciences University, Pretoria, South Africa; [2]Department of Biodiversity, University of Limpopo, Sovenga, South Africa, *E-mail: *Wilmien.powell@ul.ac.za*; [3]Electron Microscope Unit, Sefako Makgatho Health Sciences University, Pretoria, South Africa

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Summary

A new species, *Emoleptalea nwanedi* n. sp. is described from the intestine of *Schilbe intermedius*, the silver catfish or butter barbel, from the Nwanedi-Luphephe Dam in the Limpopo Province of South Africa. Fish were collected using gill nets where after they were euthanised and dissected. The parasites were sampled, fixed in 70 % EtOH and stained with Van Cleave's haematoxylin. This species represents an addition to the African cluster of *Emoleptalea* species previously described and differs from the known species due to its unique size, equal size of oral and ventral suckers, position of ovary and seminal receptacle, number of vitelline follicles and their size, as well as the unique ciliated receptors on the wall of the acetabulum. This is the first record of this parasite from the silver catfish and from southern Africa.
Keywords: trematode; *Emoleptalea*; *Schilbe*; southern Africa

Introduction

According to Jones and Bray (2008), the family Cephalogonimidae Looss, 1899 is a small family of freshwater fish parasites from Africa and Asia consisting of small, spinous digeneans in the gastro-intestinal tract of fishes, amphibians and reptiles. According to these authors its major differential character is the position of the genital pore opening at the anterior extremity of the body and the presence or absence of circumoral spines at the anterior end of the body.

The first species within this family was described by Looss in 1899 under the genus *Leptalea* Looss, 1899 which he later renamed *Emoleptalea* Looss, 1900. The parasite was described as *Emoleptalea exilis* (Looss, 1899) Looss, 1900 from the midgut of *Bagrus bayad* (Forsskăl, 1775) from the Nile River in Egypt. Dollfus (1950) described *Emoleptalea synodontis* Dollfus, 1950 from the intestine of *Synodontis notatus* Vaillant, 1893 from Lake Maka, Belgian Congo, presently known as the Democratic Republic of the Congo. Thomas (1958) followed with a description of *Emoleptalea proteropora* Thomas, 1958 from the intestine of *Clarias senegalensis* Valenciennes, 1840, presently known as *Clarias anguillaris* (Linnaeus, 1758), from the Black Volta River, Gold Coast, Ghana, West Africa. Two species were described by Shrivastava (1960) from India namely *Emoleptalea loossi* Shrivastava, 1960 and *Emoleptalea dollfusi* Shrivastava, 1960 which were both described from the intestine of *Saccobranchus fossilis* (Bloch, 1794), currently known as *Heteropneustes fossilis* (Bloch, 1794) sampled from Raipur in India.

Ramadan *et al*. (1987) described *Paramasenia rifaati* Ramadan, Saoud & Taha, 1987 from *Synodonis schall* (Bloch & Schneider, 1801) and *Synodontis serratus* Rüppell, 1829 from the Sharkiya Governorate in Egypt. This genus was later synonymised with the genus *Emoleptalea* by Jones and Bray (2008) and is now known as *Emoleptalea rifaati* (Ramadan, Saoud & Taha, 1987) n. comb.

Three species previously described under the genus *Oudhia* were synonymised with the genus *Emoleptalea* by Jones and Bray (2008). These are *Oudhia horai* Gupta, 1955 described by Gupta

* – corresponding author

(1955) from *H. fossilis* at Imphai (Manipur State), India, now known as *Emoleptalea horai* (Gupta, 1955) n. comb., *Oudhia hardayali* Kumar & Agarwal (1980) described by Kumar and Agarwal (1980) from the intestine of *Mystus vittatus* (Bloch, 1794) from Soraha Tal, Ballia, India, now known as *Emoleptalea hardayali* (Kumar & Agarwal, 1980) n. comb. and *Oudhia kanungoi* Agarwal & Agarwal, 1985 described from the intestine of *Rita rita* (Hamilton, 1822) by Agrawal and Agarwal (1985) from Uttar Pradesh and Madhya Pradesh, India, now known as *Emoleptalea kanungoi* Agarwal & Agarwal, 1985) n. comb.

In the present study, specimens of the genus *Emoleptalea* were sampled from an isolated water body, the Nwanedi-Luphephe Dam situated in the northern part of the Limpopo Province in South Africa. These specimens were sampled from the silver catfish, *Schilbe intermedius* Rüppell, 1832, commonly also known as the butter barbel.

Material and Methods

Fish was collected with gill nets from the Nwanedi-Luphephe Dam. They were euthanised with clove oil where after the intestines were examined for parasites using a Nikon Model C-Leds stereo microscope. Specimens for light microscopy were fixed in 70 % EtOH and later stained in Van Cleave's haematoxylin, routinely prepared and mounted on slides using DPX as mounting medium. All measurements were made using the NIS-Elements AR imaging (Nikon, Japan) software programme. Morphological measurements are presented in the text as follows: minimum to maximum values, followed by the average value and standard deviation in parentheses. For scanning electron microscopy specimens were fixed in 2.5 % gluteraldehyde, washed in Millonig's phosphate buffer (pH = 7.2) and dehydrated through a graded series of ethanol for one minute in each concentration. They were critically point dried (Polaron, Watford, UK), mounted on stubs, sputter coated with carbon (QT150ES, Quorum Technologies, USA) and examined with a Zeiss Supra 55 Variable Pressure Field Emission Scanning Electron Microscope (VP FE-SEM) (Carl Zeiss, Germany) at 1-2 kV.

Results

Family Cephalogonimidae
Emoleptalea nwanedi n. sp.
(Figs. 1, 2A – H, 3A – H)

Description: Diagnosis based on whole mounts of 25 mature worms and 12 worms observed with SEM: Body (Figs. 1, 2A) small, dorsoventrally flattened, measuring 582 – 722 (653 ± 55; 25) long and 320 – 407 (364 ± 33; 25) wide. Oral sucker situated subterminally at distance of 2.5 – 8.8 (5.7 ±1.9; 25) from anterior extremity of body. Sucker slightly longer than wide, measuring 91 – 123 (110 ± 11; 25) x 79 – 115 (93 ± 8; 25). Oral sucker well-developed (Fig. 2E), muscular, very stretchable, not covered with spines (Fig. 3C). Sensory receptors on wall of oral sucker consist mainly of large non-ciliated receptors (Fig. 3C). Only a few of these receptors were observed on and around oral sucker.

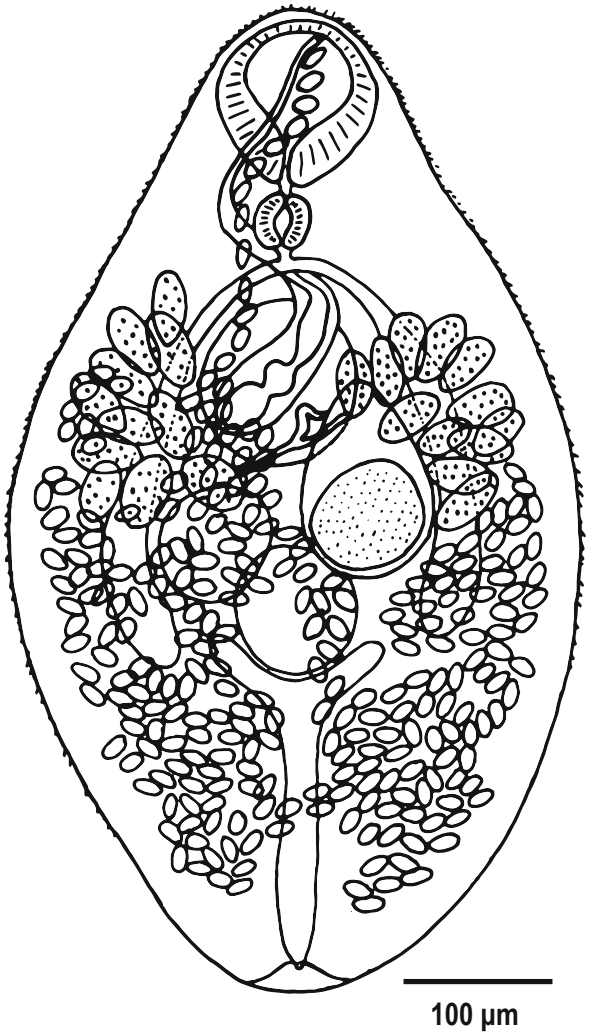

Fig. 1. Microscopic projection drawing of the adult of *Emoleptalea nwanedi* n. sp. from the intestine of *Schilbe intermedius*.

Pre-pharynx present but very short (Fig. 2E), only visible in a few specimens, measuring 5.2 – 14 (10 ± 2.4; 25) x 5.6 – 13 (10 ± 2.4; 25). It leads to small but well-developed pharynx (Fig. 2E), 30 – 43 (38 ± 4; 25) x 30 – 46 (38 ± 3.7; 25), followed by short oesophagus measuring 4.5 – 16 (10 ± 4; 25) long and 10 – 17 (13 ± 2; 25) wide. Intestinal bifurcation in forebody, caeca simple, slender and extends to end of posterior testis where they end blind, measuring 279 – 356 (319 ± 17; 25) long x 25 – 39 (31 ± 4; 25) wide.

Acetabulum (Figs. 3B,D) situated 157 – 223 (188 ± 22; 25) from anterior end of body and 59 – 99 (77 ± 16; 25) posterior to oral sucker. Almost equal in size compared to oral sucker, situated pre-equatorial and measures 90 – 123 (108 ± 11; 25) long x 95 – 125 (109 ± 9; 25) wide. Acetabulum muscular, well-formed

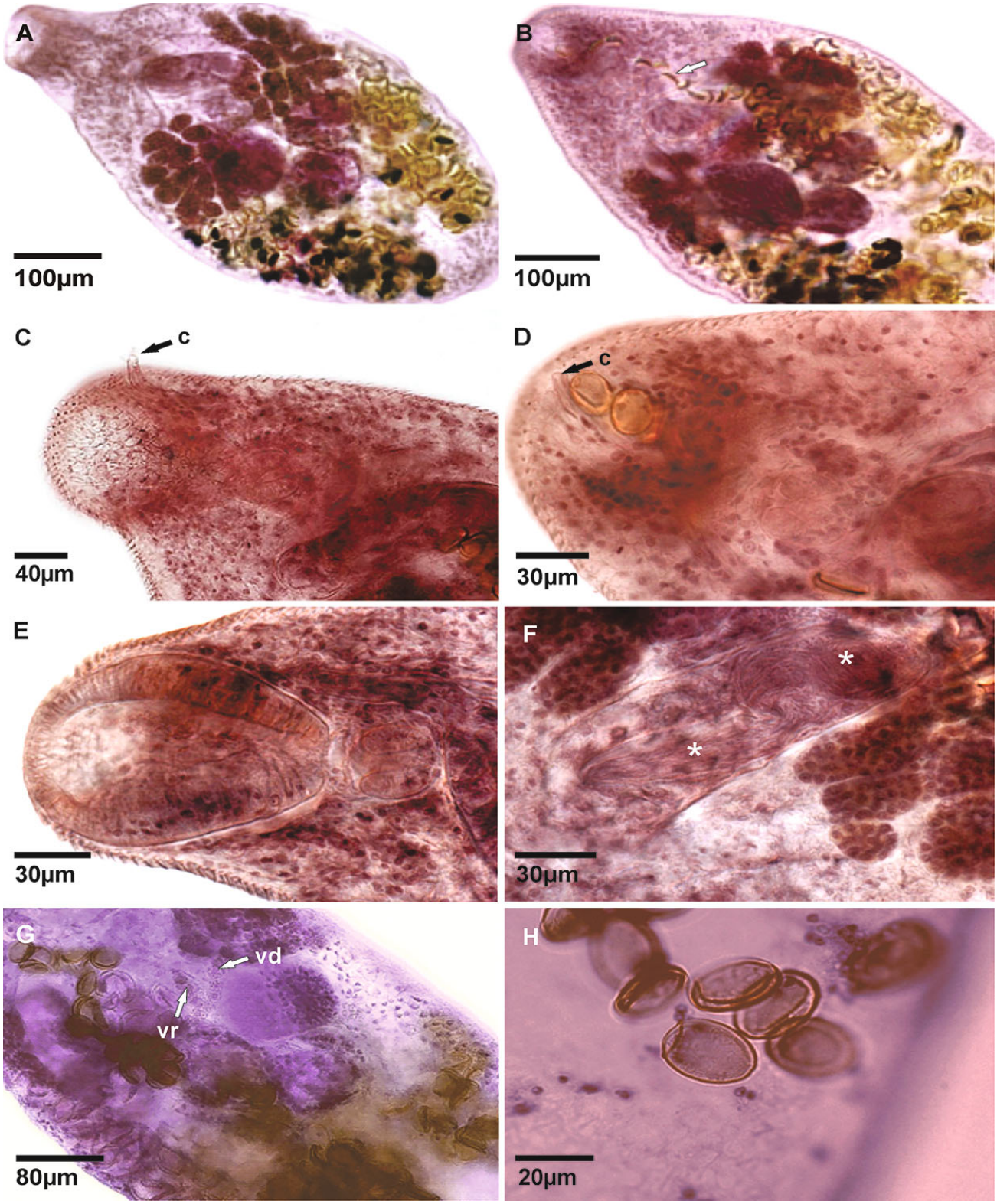

Fig. 2. Light micrographs of the adult of *Emoleptalea nwanedi* n. sp. from the intestine of *Schilbe intermedius*. 2A – whole mount; 2B – anterior half showing anteriorly directed uterus with eggs (arrow); 2C,D – cirrus opening (c) medially to oral sucker; 2E – oral sucker and pharynx separated by pre-pharynx; 2F – bipartite seminal vesicle (*); 2G – vitelline duct (vd) and reservoir (vr); 2H – operculated eggs with collar.

(Fig. 3D) and very protractible. Two types of sensory receptors observed on wall of acetabulum. First type represents bulbous receptors from which a short cilium protrudes. Second type, only found on wall of acetabulum, consists of broad plate-like base with short cilium protruding from centre of plate (Fig. 3G).

Male reproductive system consists of two testes lying oblique and intercaecal in anterior part of hindbody (Figs. 1, 2A,B). Anterior testis almost round in shape, situated 281 – 369 (317 ± 33; 25) from anterior end of body and measures 72 – 98 (85 ± 9.9; 25) in length x 75 - 101 (87 ± 9.5; 25) wide. Round posterior testis situated 310 – 379 (336 ± 27; 25) from anterior extremity of body measures 72 – 99 (84 ± 8.8; 25) long x 72 – 97 (86 ± 9.7; 25) wide. Cirrus sac elongated, claviform, sigmoid, occupying most of space between pharynx and acetabulum, sometimes overlaying acetabulum, measuring 130 – 174 (151 ± 14; 25) long x 42 - 60 (51 ± 5; 25) wide. Within it bipartite internal seminal vesicles present (Fig. 2F), consisting of posterior part measuring 49 – 89 (67 ± 13; 25) x 21 – 35 (29 ± 3.9; 25) and anterior part measuring 51 – 82 (70 ± 9.6; 25) x 16 – 24 (19 ± 2.3; 25). From here it extends into elongated pars prostatica measuring 58-94 (75 ± 11; 25) x 17 – 35 (25 ± 5; 25), followed by long ejaculatory duct, 124 – 149 (138 ± 7; 25) x 9.8 – 12.8 (10.8 ± 0.8; 25), that extends to lateral edge of oral sucker where it opens as a genital pore (Figs. 2C,D). Extending cirrus is not covered with spines.

Female reproductive system consists of round to slightly oval ovary, measuring 96 – 123 (111 ± 10; 25) in length and 79 – 101 (90 ± 7; 25) wide. Ovary situated almost in middle of body, submedian, but overlays edge of anterior testis (Figs. 1, 2B). Ovary situated 219 – 322 (263 ± 38; 25) from anterior end of body. Seminal receptacle large, sometimes overlaying ovary, situated 253 – 302 (274 ± 19; 25) from anterior end of body. This sperm filled structure measures 61 – 91 (76 ± 11; 25) in length and 46 – 88 (58 ± 9.7; 25) wide. Uterus coils mainly in post-testicular region, descends on the right to close to posterior end of body where after it ascends on the left again, occupying most of hindbody. Eggs are numerous and small, measuring 21 – 24 (23 ± 0.9; 25) long x 13 – 16 (14 ± 0.9; 25) wide. Eggs are operculated forming a slight shoulder edge at opposite ends of opercule line (Fig. 2H).

Vitelline follicles in lateral fields are grouped together forming a cluster of 10 – 14 (12 ± 1.1; 25) follicles (Fig. 2A) on both sides of body on either side of acetabulum to half way of anterior testes flowing into a vitelline reservoir (Fig. 2G) before discharging into ootype. Individual follicles measure 42 – 61 (51 ± 6.5; 25) long x 30 – 42 (36 ± 3.9; 25) wide.

Excretory vesicle "Y"-shaped consisting of elongated stem measuring 131 – 172 (155 ± 13; 25) long x 26 – 45 (35 ± 5.9; 25) wide, followed by anterior parts measuring 63 – 104 (85 ± 14; 25) long x 15 – 27 (19 ± 3; 25) wide. Pore opens terminally at posterior end of body (Fig. 3A).

Tegument of anterior part of forebody covered by scale-like spines (Fig. 3E) which are embedded in tegument all around oral and ventral suckers (Fig. 3B). These spines are densely packed, posteriorly directed and can retract into body. Posterior to acetabulum these spines are more sharply pointed and seemingly arranged in rows (Fig. 3F). From anterior part of hindbody, these spines decline in number and concentration towards the posterior end where body is only covered by a rough spine-less tegument (Fig. 3A). Enlarged circumoral spines absent. Very few sensory receptors present elsewhere on body of the worm. A few large non-ciliated receptors were observed randomly on body (Fig. 3C), whereas a few small bulbous ciliated receptors and longer ciliated receptors (Fig. 3H) were observed in the area between the two suckers.

Seventy-one specimens of *S. intermedius* were sampled and examined for parasites. Only 29 of these were found to harbour *Emoleptalea* parasites, resulting in a prevalence infection of 40.8 %. A total number of 1468 parasites were collected. The mean intensity was calculated as 50.6 and the mean abundance as 20.7.

It also became apparent that the size of *S. intermedius* specimens collected had an effect on the number of parasites that possibly could be sampled. Small fish, less than 16 cm, were seldom infected, while sizes ranging between 16 to 25 cm yielded more parasites, but specimens larger than 25 cm were infected with the most parasites. This data is summarised in Table 1.

Table 1. Infection statistics of *Schilbe intermedius* with *Emoleptalea nwanedi* n. sp. regarding the size of the host vs. the number of parasites found.

Length of sampled fish	Prevalence (%)	Abundance	Intensity	Intensity range
< 16 cm	14.2	1.6	11.5	2 – 14
16 – 25 cm	48	8.9	18.5	2 – 147
> 25 cm	56	39.8	70.1	1 – 327

Taxonomic summary

Type and only known host: *Schilbe intermedius* Rüppell, 1832.
Type and only locality: Nwanedi-Luphephe Dam, Limpopo Province, South Africa (22°38'36"S 30°24'29.5"E).
Site of infection: Duodenum.
Prevalence of infection: 40.8 %.
Specimens deposited: Deposited in the Aquatic Parasitology Research Group, Department of Biology, Sefako Makgatho Health Sciences University, Pretoria, South Africa. Adult specimens: holotype: 2011/03/15/01; voucher specimens: 2012/02/29/01-16.
Etymology: The species epithet, *nwanedi*, derived from the type locality, an ecological jewel in the Limpopo River System.

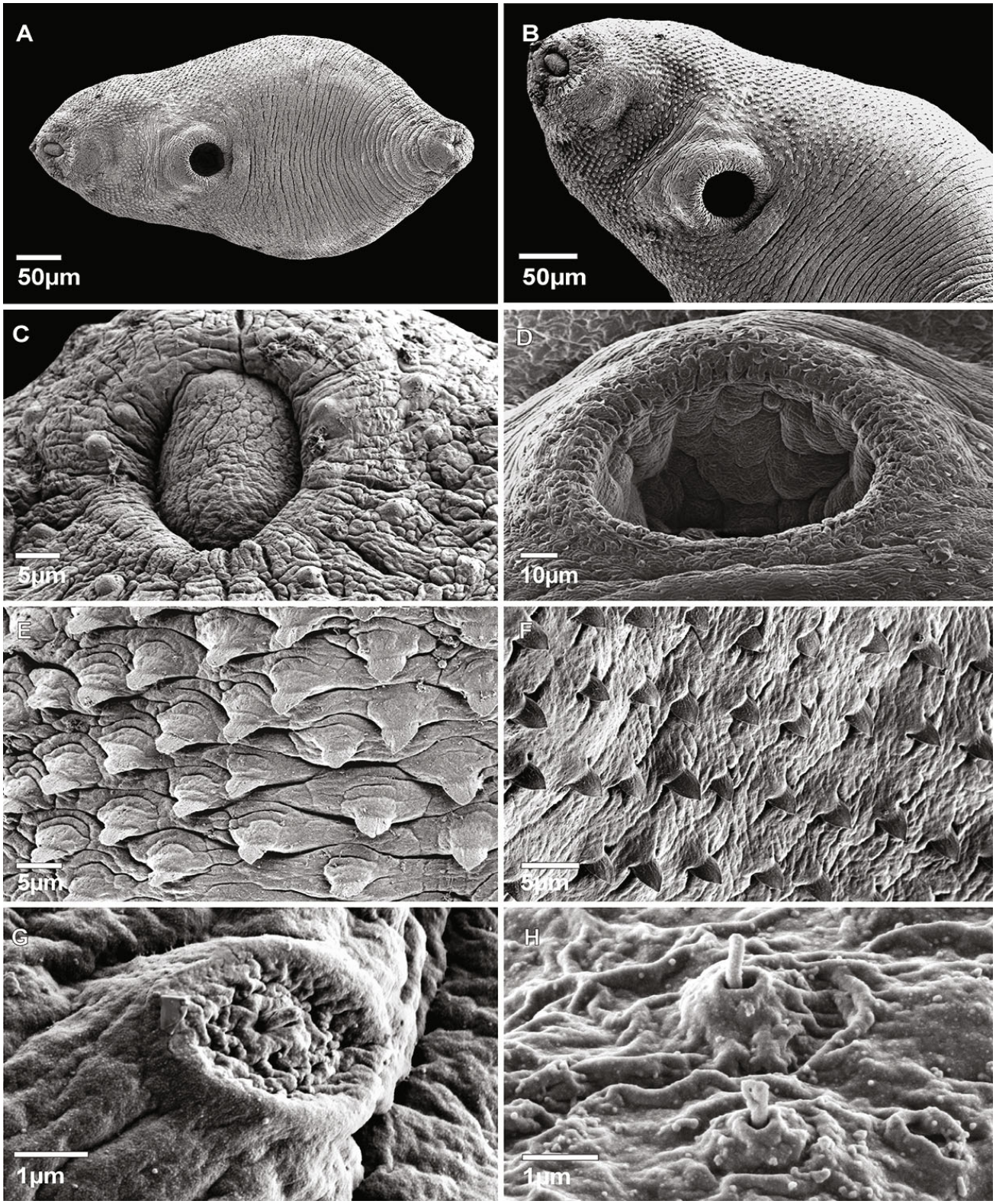

Fig. 3. Scanning electron micrographs of the adult of *Emoleptalea nwanedi* n. sp. from the intestine of *Schilbe intermedius*. 3A – whole mount; 3B – anterior half showing body spines; 3C – oral sucker showing non-ciliated receptors; 3D – acetabulum showing receptors; 3E – tegumental features between oral sucker and acetabulum; 3F – body covering posterior to acetabulum; 3G – enlarged plate-like receptors on acetabulum edge; 3H – longer ciliated receptors on body and around suckers.

Discussion

From the introduction it is clear that species within the genus *Emoleptalea* can be clustered into two groups, those described from Africa and those from India. The Indian group now includes five species namely *E. horai, E. loossi, E. dollfusi, E. hardayali* and *E. kangungoi.* All five of these species make use of catfish as their definitive host. *Emoleptalea loossi*, *E. dollfusi* and *E. horai* were found in the Asian stinging catfish (*Heteropneustes fossilis*) (Shrivastava, 1960; Gupta, 1955), *E. hardayali* in the striped dwarf catfish (*Mystus vittatus*) (Kumar & Agarwal, 1980) and *E. kanungoi* in the striped catfish (*Rita rita*) (Agarwal & Agarwal, 1985).

The African group also includes five species namely *E. exilis, E. synodontis, E. proteropora, E. rifaati* and *E. nwanedi* n. sp. Of these only *E. proteropora* is found in a *Clarias* species, namely *Clarias anguillaris* (Thomas, 1958). *Emoleptalea exilis* was described from the black Nile catfish, *Bagrus bayad* (Looss, 1899) Looss, 1900. The remaining three species were all described from squeakers: *E. synodontis* from the one-spot squeaker, *Synodontis notate* (Dollfus, 1950), *E. rifaati* on the other hand, was described from two squeaker species, namely the Nile squeaker, *Synodontis schall* and the shield-head squeaker, *Synodontis serratus* (Ramadan, Saoud & Taha, 1987). The newly described *E. nwanedi* n. sp. however, was described from a siluriforme fish, the silver catfish *S. intermedius*.

The species material was collected over a period of three years during three different field trips resulting in the size ranges as indicated in the results. The present species show minor morphological differences compared to the four *Emoleptalea* spp. described from Africa and the five species described from India. The following five differences indicate the present species to differ from the nine known species: the present species is the smallest of all the *Emoleptalea* species described from Africa and India to date. The oral sucker of the present species is more or less of equal size to the acetabulum, compared to the already described species that indicates that the suckers are not uniform in size. The ovary in the present species most often overlays the anterior testis, compared to other species that indicate the ovary to be a distance away. The large seminal receptacle most often overlays the ovary not as indicated for other described species. The vitelline follicles are of greater numbers and size compared to the rest of the African species, and even more so for the Indian species.

This study also represents the first description of an *Emoleptalea* species using scanning electron microscopy. These results clearly show the body to be covered with triangular spines that are arranged anteriorly in rows. Another unique feature is the ciliated receptors on the acetabulum that protrude from a plate-like base. Electron microscopy also shows that the cirrus is not covered with spines.

The only related genus of digeneans from African and Asian freshwater fishes that appears morphologically similar to *Emoleptalea* in the Cephalogonimidae, is *Masenia* Chatterji, 1933. Two species have previously been described from Africa, i.e. (i) *Eumasenia bangweulensis* Beverley-Burton 1962 described from *Clarias mellandi* (synonymised with *Clarias ngamensis*) from the Mangweula Swamps in northern Rhodesia (now Zimbabwe) (Beverley-Burton, 1962) and characterised by 48 circumoral spines and vitelline follicles that occur in two groups on either side of the ventral sucker; (ii) *Eumasia synodontis* Khalil & Thurston 1973 described from *Synodontis victoriae* from Jinja on Lake Victoria, Uganda (Khalil & Thurston, 1973), with 36 – 40 circumoral spines and vitelline follicles that extend from the level of the ventral sucker to the level of the margin of the posterior testis. Jones and Bray (2008) synonymised *Eumasenia* with *Masenia*, but these species differs from *Emoleptalea* species by a crown of circumoral spines at the anterior end of the body and vitelline follicles that are mainly located between the ventral sucker and the level of the testes.

This is the first description of an *Emoleptalea* species from southern Africa and from *S. intermedius*. Follow-up studies were also attempted in order to try and find the intermediate hosts. Two freshwater snail species were collected. *Lymnaea natalensis* Krauss, 1848 was found to secrete 27-spined echinostome cercariae, and *Biomphalaria pfeifferi* (Krauss, 1848) was found to secrete strigeid cercariae, categorised as the pharyngeal, distome, longifurcate type. The metacercarial stage also remains unknown, but it is suspected that the larval stage of the parasite utilises smaller fish species as second intermediate hosts.

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Case Report

Filaroidosis infection in an immunocompetent adult dog from France

M. CERVONE[1]*, A. GIANNELLI[2], D. ROSENBERG[3], S. PERRUCCI[4], D. OTRANTO[2]

[1]*Small Animal Veterinary Clinic Paris III, Bl des Filles du Calvaire 75003 Paris, France, E-mail: *mariocervone@live.it*;
[2]Department of Veterinary Medicine, University of Bari, Str. prov. per Casamassima km 3, 70010 Valenzano (Bari) Italy,
E-mail: *giannelli.alessio@gmail.com*, *domenico.otranto@uniba.it*; [3]Micen Vet Centre, 58 Rue Auguste Perret, Parc Technologique,
Europarc, 9400 Créteil, France, E-mail: *d.rosenberg@micen-vet.fr*; [4]Department of Veterinary Science, University of Pisa,
Viale delle Piagge 2, 56124 Pisa, Italy, E-mail: *stefania.perrucci@unipi.it*

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Summary

A dog from Paris (France) was referred with a 2-week history of dry cough, intermittent acute onset of dyspnoea, and acute abdominal pain. A generalised bronchoalveolar infiltrate with a patchy distribution was observed at chest x-rays and computed tomography (CT) scans. Negative results were obtained through several faecal examinations for cardiorespiratory nematodes by using the Baermann technique and at two blood analysis with a commercially available test for the detection of *A. vasorum* antigen (the first one at the first visit and second one at the control visit, one month later). PCR methods for the identification of *A. vasorum* and *C. vulpis* were also accomplished. At the control visit, nematode L1s were found during direct microscopic examination of bronchoalveolar lavage fluid (BALF). Thus, a different antigen-based assay for the detection of *A. vasorum* was performed with a positive result. Moreover, based on morphology, isolated larvae were identified as *Filaroides hirthi*. The dog was treated with fenbendazole (50 mg/kg per os once daily) for two consecutive weeks. After five months, the dog was referred again for the intermittent acute onset of dyspnoea and was found to be still positive for *F. hirthi* larvae at BALF examination. A 15-day treatment regimen with fenbendazole in combination with three subcutaneous injections of ivermectin (0.4 mg/kg, once every two weeks), was then performed. No larvae were detected at two BALF microscopical examinations performed one month apart. Results from this case report underline the importance of including *F. hirthi* infections in the differential diagnosis of dog bronchopneumonia.
Keywords: *Filaroides hirthi*; canine verminous bronchopneumonia; France

Introduction

Reports of nematodes parasitizing the respiratory tract of carnivores are increasingly common in Europe (Traversa *et al*., 2010; Giannelli *et al.*, 2017) and these parasites can cause severe and occasionally fatal impairment (Traversa *et al*., 2010). Of the nematode species affecting dogs, *Angiostrongylus vasorum* is the most common (Morgan & Shaw, 2010; Helm *et al*., 2010), whereas *Crenosoma vulpis*, *Oslerus osleri* and *Filaroides hirthi* show more limited geographic distribution (Traversa *et al*., 2010; Latrofa *et al*., 2015). In particular, *Filaroides hirthi* has been sporadically documented in dogs from Germany (Bahnemann & Bauer, 1994), Great Britain (Spencer *et al*., 1985), Australia (Beveridge *et al*., 1983), Japan (Kagei *et al*., 1976), United States (Rubash, 1986; Pinckney *et al*., 1988), Ireland (Torgerson *et al*., 1997), France (Bordeau & Ehm, 1992) and Spain (Caro-Vadillo *et al*., 2005). Unlike other dog metastrongylid nematodes, *F. hirthi* has a direct life cycle. Puppies are infected through ingestion of first-stage larvae (L1s) passed by the faeces of chronically infected bitches (Georgi *et al*., 1979). L1s rapidly make their way to the lungs via the he-

* – corresponding author

patic-portal or mesenteric lymphatic system and can survive within the mesenteric lymph nodes for extended periods, thus exposing the animal to auto-reinfection (Georgi *et al.*, 1979). Once in the respiratory apparatus, larvae moult into adults and within around five weeks, females shed larvae that can be detected in the faeces of the infected host (Bowman, 2000). Adults, causing severe bronchopneumonia, can remain hidden in lung parenchyma for long periods. Clinical disease outcome has most often been diagnosed in stressed young dogs, especially of toy breeds. However, severe clinical presentations can also be observed in immunocompetent and immunocompromised adults (Caro-Vadillo *et al.*, 2005; Conboy, 2009). Canine *F. hirthi* infection is usually marked by dry cough (Bowman, 2000) along with rapid breathing, dyspnoea, and exercise intolerance (Rubash, 1986; Andreasen & Carmichael, 1992; Bourdeau & Ehm, 1992). The diagnosis of the infection is based on direct detection of L1s in bronchoalveolar lavage or in the faeces (Pinckney *et al.*, 1988; McGarry & Morgan, 2009). However, due to intermittent faecal larval shedding and the occurrence of auto-reinfections, the diagnosis and treatment of canine *F. hirthi* infection remain challenging (Bauer & Bahnemann, 1996).

Case presentation

A seven-year old unspayed female West Highland white terrier living in Paris (France) was admitted to the Small Animal Veterinary Clinic Paris III (Paris, France) for decreased appetite, acute abdominal pain, dry cough, and intermittent acute onset of dyspnoea. Previous history included cranium-mandibular osteopathy at the age of 10 months, successfully treated with corticosteroids. The dog had been purchased at the age of three months in Ireland and since than had never travelled out of France. Physical examination revealed severe abdominal pain at the cranial abdominal region, tachypnoea (50 breaths/min) and bradycardia (60 bpm), associated with respiratory sinus arrhythmia. Thoracic auscultation disclosed pronounced bilateral wheezing and crackles, along with increased breathing sounds in trachea. A complete blood count showed moderate anaemia (Hgb 12.83 g/dl; reference interval 13.2 – 19.2), moderate leucocytosis ($13.7x10^9$/L; reference interval 6 – 13) associated with eosinophilia ($2.4x10^9$/L, reference interval 0.0 – 1.2) and thrombocytopenia ($20x10^9$/L; reference interval 150 – 500). Blood smear examination highlighted the presence of giant platelets, while the coagulation profile was within the normal reference range. No biochemical abnormality was found at blood and urine analysis, and no abdominal malformations or abnormalities were recorded by either ultrasonography or abdominal computed tomography (CT). Chest x-rays revealed an extensive bronchoalveolar infiltrate with a patchy distribution (Fig. 1), what was also confirmed by CT (Fig. 2A). Echocardiography revealed no defects, while bronchoscopy showed tracheal and bronchial hemorrhagic areas associated with mucosal hyperaemia. Cytology of the broncho-alveolar lavage fluid (BALF) showed moderate cell density, characterised by high levels of eosinophils (56 %) and neutrophils (22 %) and few macrophages (22 %).

On the basis of these findings, the main differential diagnoses included nematode, mycotic, allergic and inflammatory bronchopneumonia, as well as idiopathic pulmonary fibrosis, primary or

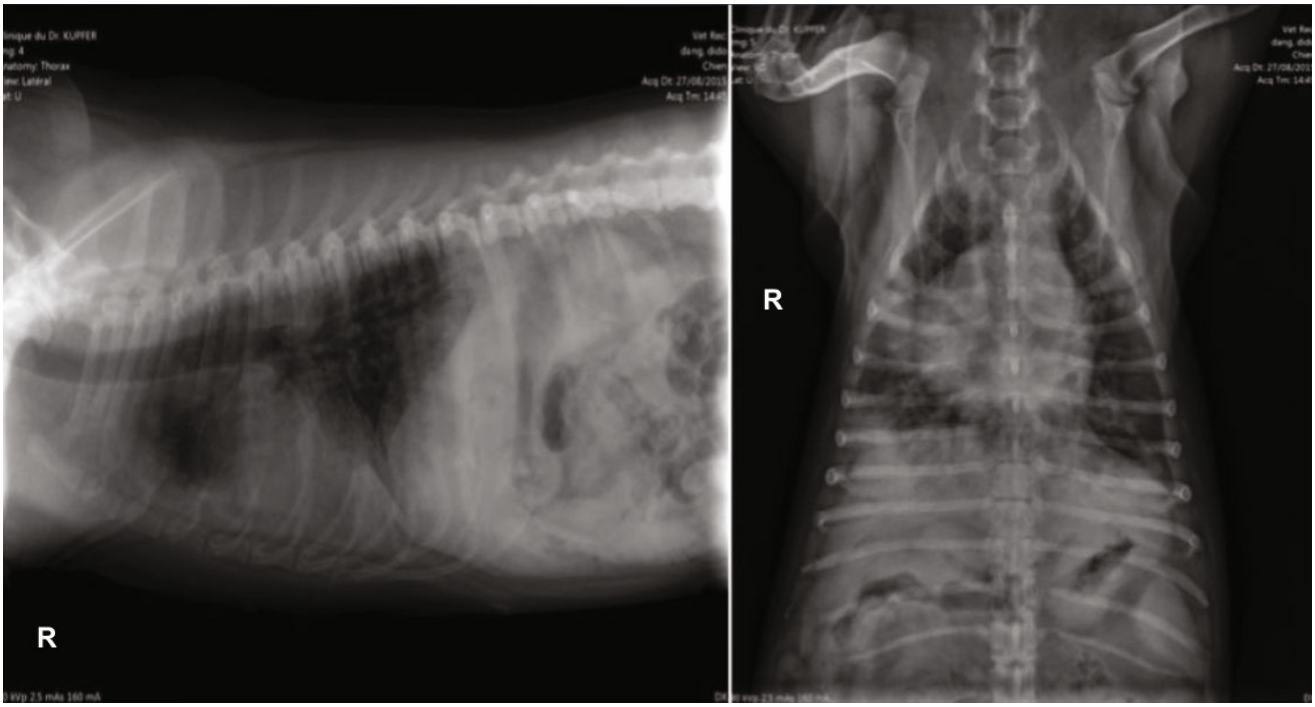

Fig. 1. Right lateral (on the left) and ventrodorsal (on the right) thoracic radiographs of the examined dog showing an extensive broncho-alveolar infiltrate and consolidated areas with a patchy distribution. Lesions are more severe in the right hemithorax.

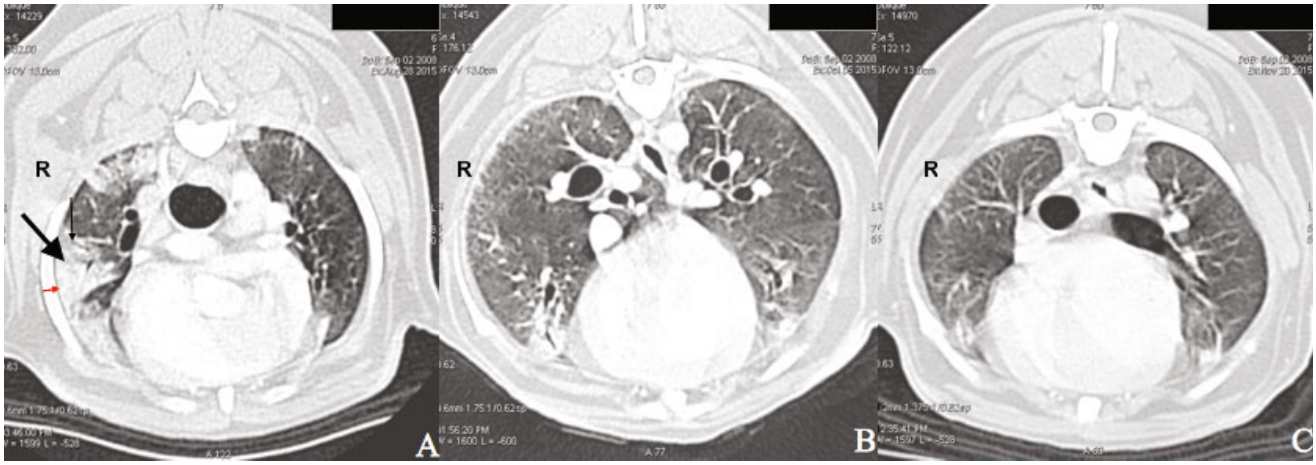

Fig. 2. Transverse computed tomographic images of the lungs of the dog. (A) Pulmonary lesions at clinical presentation, showing a large consolidated area (black thick arrow) with formation of air bronchograms (black thin arrow), surrounded by diffuse zones of ground-glass opacification (red thin arrow). Lesions are more severe in the right hemithorax (B) Moderate improvement in pulmonary lesions after few days of treatment. (C) Improvement in pulmonary lesions 15 days after starting treatment with oral fenbendazole. Consolidated areas have almost disappeared.

metastatic pulmonary neoplasia, granulomatous pneumonia and pulmonary granulomatosis. Parasitological analysis on faecal samples collected over three consecutive days and examined as fresh smears, by the Baermann technique and by flotation test using a low-density flotation solution (specific gravity 1.2), proved negative for parasites. Negative results were also obtained after blood analysis with a commercially available blood test (Angio Detect™, IDEXX, Westbrook, USA) for the detection of *A. vasorum* antigen and also after faecal samples analysis with PCR for the identification of *A. vasorum* and *C. vulpis* (performed by IDEXX Laboratories, France). Bacteriological examination of BALF revealed the presence of extra-cellular *Pseudomonas* species. Real-time PCR on BALF was negative for *Toxoplasma gondii* and *Pneumocystis carinii* (Biomnis laboratories, France). Idiopathic eosinophilic bronchopneumonia was suspected and the dog was treated with marbofloxacine (4 mg/kg/day, Marbocyl®; Vetoquinol S.A.) during 15 days and prednisolone (10 mg/kg/day, Dermipred®; Sogeval) during 30 days. Treatment resulted in moderate improvement in clinical condition and thoracic pulmonary lesions by CT examination (Fig. 2B) at control visit, performed one month later. In contrast, the BALF control cytological analysis, performed 30 days after the beginning of the treatment, revealed absolute neutrophilia (43 %) with normal eosinophilia (3 %) associated with a large number of nematode L1s found at direct microscopic examination of BALF. The suspicion that at the first visit the dog had an *A. vasorum* infection in the prepatent period, led us to repeat the antigen blood test (Angio Detect™, IDEXX, Westbrook, USA) and the Baermann test at the control visit. Both these tests were negative for a second time. Therefore, a different antigen-based assay (Schnyder *et al*., 2011) with a positive result for the detection of *A. vasorum* was performed. Additionally, based on morphology and dimensions according to previously reported data (McGarry & Morgan, 2009) the L1s found in BALF were microscopically identified at the species level. The collected L1s measured 265 ± 13µm and were characterised by a straight tail with a single slight dorsal indentation, ending in a lance-like shape (Fig. 3). Larvae isolated from faecal samples and BALF were also subjected to molecular identification using primers targeting partial 12S and 18S rRNA genes (Fila_12SF: 5'-CGGGAGTAAAGTTTTGTTTAAACCG-3' and Fila_12SR: 5'- CATTGACGGATGGTTTGTACCAC-3'; NC18SF1: 5'-AAAGATTAAGCCATGCA-3' and NC5BR: 5'-GCAGGTTCACCTACAGAT-3', respectively) and run PCR protocol described elsewhere (Latrofa *et al*., 2015). Although both genes offer useful insights into the identification of various nematode species (Hu *et al*., 2004; Petterson-Kane *et al*., 2009; Brianti *et al*., 2012), the amplification turned out unsuccessful likely due to the contamination by bacterial and fungal DNA (Jefferies *et al*., 2010). However, based on the morphological features of the larvae and clinical signs a diagnosis of *F. hirthi* infection was confirmed and the dog was treated with oral fenbendazole once daily (50 mg/kg; Panacur™, MSD Animal Health Srl, France) for two consecutive weeks (Rubash, 1986). Fifteen days after the start of the treatment, the owner reported improvement in respiratory signs, and repeated BALF cytological analysis showed blood cell characteristics (neutrophils 22 %, eosinophils 0 %, macrophages 73 % associated with haemosiderophages) and confirmed the absence of L1s at direct microscopic examination. Thoracic CT showed excellent improvement of the pulmonary lesions (Fig. 2C). Based on its efficacy against the immature stages and the reduction of infection levels of other cardio-respiratory nematodes, milbemycin oxyme (at 0.75 mg/kg, Trifexis®, Elanco Animal Health) was then administered once monthly *per os* to prevent reinfections and auto-reinfections (Conboy *et al*., 2013a; Böhm *et al*., 2014; Lebon *et al*., 2016).

Five months later, the dog was referred again for the intermittent acute onset of dyspnoea. Chest CT showed a relapse of alveolar

Fig. 3. *Filaroides hirthi* first stage larva detected at the microscopic examination of the BALF (40x magnification). Note the straight tail with a single slight dorsal indentation (thick arrow), ending into a lance-like shape (thin arrow), consistent with *F. hirthi*.

opacities with patchy distribution, and direct microscopic examination of BALF revealed once more the presence of live *F. hirthi* L1s. Thus, fenbendazole treatment (50 mg/kg per os for two weeks) combined with three subcutaneous off-label administrations of ivermectin (0.4 mg/kg, once every two weeks; Ivomec®; Merial), were performed. One month later, the thoracic CT showed normal lung patterns, and no larvae were detected at two BALF microscopical examinations performed one month apart.

Discussion

The genus *Filaroides* includes ovoviviparous nematodes that localise in the respiratory system of dogs and wild canids. Dog infections with *Filaroides* species are considered relatively uncommon, possibly because many infected dogs are clinically asymptomatic (Caro-Vadillo et al., 2005; Caswell and Williams, 2007). Indeed, clinical diseases caused by *F. hirthi* have been generally associated with immunocompromised, stressed or young dogs (Caro-Vadillo et al., 2005). Since the literature contains only case reports, the prevalence of *F. hirthi* infection in Europe and in other geographical areas is unknown (Bauer & Bahnemann, 1996; Caro-Vadillo *et al.*, 2005). Besides other than the frequent chronic and sub-clinical infections, this could be due to diagnostic difficulties resulting from the intermittent shedding of *F. hirthi* larvae in the faeces of infected animals. This is probably also caused by wrong identification of *F. hirthi* larvae that can be confused with the most common *A. vasorum* species. Since the larvae of *Filaroides* spp. larvae are lethargic and therefore do not migrate out of the faeces easily (Traversa *et al.*, 2010) their detection in faecal samples examined by the Baermann method may be unlikely. However, the geographical distribution of *F. hirthi* could be truly limited, resulting in sporadic infections throughout the world. In the dog examined herein, *F. hirthi* L1s were identified at BALF microscopic examination. Based on their significantly smaller size and on their tails showing a notch followed by a constriction and a terminal lance-like end, without any kink, undulation or spine, isolated larvae were distinguished from those of *A. vasorum* showing a prominent dorsal spine and a double cuticle indentation at the caudal end (McGarry & Morgan, 2009; Traversa *et al.*, 2010; Taylor *et al.*, 2007). The different morphology of the caudal end also allowed their differentiation from L1s of *C. vulpis*, showing a straight and uniformly pointed tail (McGarry & Morgan, 2009; Traversa *et al.*, 2010). The L1s of *F. hirthi*, *Filaroides milksi* and *Oslerus* (*Filaroides*) *osleri* are morphological identical and cannot be differentiated from each other (Traversa *et al.*, 2010; Conboy, 2009). However, in the dog examined, characteristic *O. osleri* tracheobronchial nodules were not evidenced by bronchoscopy. On the other hand, dog *F. milksi* infection has been rarely reported in Europe (Cremers *et al.*, 1978). Moreover, the validity of *F. milksi* and *F. hirthi* as two separate species has been questioned (Conboy, 2009).

Canine *F. hirthi* infection is often subclinical in healthy and immunocompetent dogs. Nevertheless fatal respiratory disease outcome has been reported after corticosteroid treatments or because of other immunosuppressive conditions, including chronic stress,

generalized demodicosis or adrenal cortical carcinoma (Bauer & Bahnemann, 1996). In aged dogs, the infection can begin as non-productive cough, sometimes associated with poor general condition and acute or progressive dyspnoea or tachypnoea (Bowman, 2000; Torgerson *et al*., 1997).

Similarly to *A. vasorum* infection (Martin *et al*., 1993), the abdominal pain observed in the dog herein examined could be related to L1s migration through the mesenteric lymph nodes, liver or kidney or as a result of pleural or diaphragmatic inflammation. Moreover, a mixed pulmonary pattern affecting all the lung lobes, mainly characterized by bronchiolitis, peribronchitis and perivasculitis, focal or interstitial pneumonia, granulomatous lesions and pleural fibrosis with a predominance of interstitial and alveolar infiltration, is the most common radiographic finding for *F. hirthi* infections (Bowman, 2000). As described in the case here reported, free larvae in the alveolar lumen may induce an inflammatory reaction characterized by numerous neutrophilic granulocytes, while eosinophilia can be observed in peripheral blood (Bahnemann & Bauer, 1994). Negative faecal examinations results do not exclude infection by *Filaroides* species (Caro-Vadillo *et al*., 2005), where bronchial or tracheal washing are more reliable than coprology in the detection of L1s (Brownlie, 1990). Thus, *F. hirthi* larvae are detected most accurately by the examination of bronchial mucus (Conboy, 2009). In the present case, the Baermann method was used in association with BALF direct microscopic examination, as well as serological and molecular detection methods in order to rule out *A. vasorum* and *C. vulpis* infection.

The prevalence of *A. vasorum* is high in France and this cardio-respiratory nematode should be always considered in the differential diagnosis of dog bronchopneumonia. Although for the positive commercial *A. vasorum* sandwich-ELISA used herein a specificity of 94 % has been reported (Verzberger-Epshtein *et al*., 2008; Schnyder *et al*., 2011), a possible cross-reactivity with *F. hirthi* has not previously been assessed. Based on the results obtained, the cross-reactivity of this immunological *A. vasorum* diagnostic test with *F. hirthi* should not be ruled out. Since this test can reveal the presence of *A. vasorum* antigens until 34 days after the treatment and it is always positive in dogs harbouring only one worm (Schnyder *et al*., 2011) the occurrence of a previous *A. vasorum* infection in dog examined in this study cannot be excluded. Considering the high prevalence of *A. vasorum* infection in France and in other European countries (Lebon *et al*., 2016; Lempereur *et al*., 2016; Traversa & Guglielmini, 2008), great attention is thus required for interpretation of results and diagnostic procedures.

For the treatment of dog *Filaroides* infections, the effective use of fenbendazole (50 mg/kg oral once a day for 10 to 14 days), albendazole (25 to 50 mg/kg twice a day for five consecutive days repeated two weeks later), and single administration of injectable ivermectin at 0.4 – 1 mg/kg, has been reported in previous studies (Bauer & Bahnemann,1996; Bowman, 2000; Caro-Vadillo *et al*., 2005). The treatment of *F. hirthi* is particularly challenging because this parasite does not require an intermediate host for its development, and reinfections and auto-reinfections frequently occur (Georgi *et al*., 1979; Torgerson *et al*., 1997). This was the main reason why milbemycin oxime and ivermectin treatments were performed in this case report. However, the treatment with oral milbemycin oxime was unsuccessful from preventing the *F. hirthi* infection. The efficacy of milbemycin oxime for the prevention of *A. vasorum* and the reduction of *A. vasorum* and *C. vulpis* infection levels, have been evidenced (Conboy *et al*., 2013; Böhm *et al*., 2014; Lebon *et al*., 2016). However, dosing intervals for the treatment of infections and the prevention in clinical disease have not yet been established (Conboy *et al*., 2013a). Moreover, for the effective treatment of other respiratory nematodes, as *E. boehmi* infection, milbemycin oxime should be used at the increased dose of 2 mg/kg (Conboy *et al*., 2013b; Cervone *et al*., 2017). All these factors might represent possible reasons for the failure of milbemycin oxime in the case study reported here. Though moxidectin larvicidal and adulticidal activity against *A. vasorum* in dogs has been demonstrated (Willesen *et al*., 2007; Schnyder *et al*., 2009), in France moxidectin is available only as a topic spot-on formulation for its use in companion animals. Since the patient examined here had previously showed a cutaneous reaction to spot-on formulations, the prophylactic use of oral milbemycin oxime was preferred to moxidectin in this case report. Although the use of ivermectin should be discouraged in canine medicine, unless mandatory due to the lack of other efficacious drugs, injectable ivermectin was preferred in this study because of previous reports on its effectiveness for the treatment of *F. hirthi* in dogs (Erb & Georgi, 1982; Pinckney *et al*., 1988; Bauer & Bahnemann, 1996).

Based on the resolution of respiratory signs and the absence of L1s at two BALF microscopical examinations performed one month apart following the combined fenbendazole and ivermectin treatment, it can be assumed that the dog from the case here presented was healed from *F. hirthi* infection. However, due to lack of follow-up of the dog examined in this case report, further reinfections after this combined treatment cannot be ruled out.

In conclusion, results from this study underline the importance of including *F. hirthi* infections in the differential diagnosis of dog bronchopneumonia.

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Research Note

The first record of the invasive Asian fish tapeworm (*Schyzocotyle acheilognathi*) from an endemic cichlid fish in Madagascar

T. SCHOLZ[1,*], A. ŠIMKOVÁ[2], J. RASAMY RAZANABOLANA[3], R. KUCHTA[1]

[1]Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic, E-mail: **tscholz@paru.cas.cz*; *krtek@paru.cas.cz*; [2]Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic, E-mail: *simkova@sci.muni.cz*; [3]Department of Animal Biology, Faculty of Science, University of Antananarivo, BP 906 Antananarivo 101, Madagascar, E-mail: *jeanne.rasamy@gmail.com*

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Summary

The Asian fish tapeworm, *Schyzocotyle acheilognathi* (Yamaguti, 1934) (Cestoda: Bothriocephalidea), is an invasive parasite of freshwater fishes that have been reported from more than 200 freshwater fish worldwide. It was originally described from a small cyprinid, *Acheilognathus rombeus*, in Japan but then has spread, usually with carp, minnows or guppies, to all continents including isolated islands such as Hawaii, Puerto Rico, Cuba or Sri Lanka. In the present account, we report the first case of the infection of a native cichlid fish, *Ptychochromis* cf. *inornatus* (Perciformes: Cichlidae), endemic to Madagascar, with *S. acheilognathi*. The way of introduction of this parasite to the island, which is one of the world's biodiversity hotspots, is briefly discussed.
Keywords: Invasive parasite; new geographical record; Cestoda; Cichlidae; Madagascar

Introduction

Madagascar is a biodiversity hotspot with over 90 % of its fauna represented by endemic species (Hobbes & Dolan, 2008). Even though the island is situated near the African continent, it originally belonged to the Indian Peninsula, from which it split around 88 million years ago (Rosemary & Clague, 2009). The island's diverse ecosystems and unique wildlife are threatened by the encroachment of the rapidly growing human population and other environmental threats, including water pollution (Benstead *et al.*, 2003). As much as 41 % (71 from 172) species of freshwater fish from Madagascar are endemic, including two endemic families (Froese & Pauly, 2017). Native fish are also endangered by the introduction of non-native fish species and their parasites, which may have a detrimental effect on local fish communities, especially of native hosts, which are not adapted to these newly introduced parasites (Lévêque, 1997).

One of the most widely distributed fish parasites, and probably the most successful invasive parasite at all, is the so called Asian fish tapeworm, *Schyzocotyle acheilognathi* (Yamaguti, 1934) (syn. *Bothriocephalus acheilognathi*) (Cestoda: Bothriocephalidea). This tapeworm of the supposedly East Asian origin has been reported from more than 200 species of freshwater fish throughout the world, including isolated islands such as the Hawaii, Puerto Rico, Cuba or Sri Lanka (Font, 2003; Choudhury *et al.*, 2006; Scholz *et al.*, 2012).

The parasite has been disseminated worldwide mainly due to the import of veterinary uninspected common carp, grass carp, guppies and minnows (Scholz *et al.*, 2012). It may reduce the growth of fish fry or even cause mortality, as reported in the 1960' – 1980's in the former USSR and central Europe (Bauer *et al.*, 1973; Scholz, 1999). However, it also represents a threat for populations of native fish (Williams & Jones, 1994; Dove *et al.*, 1997).

Material and Methods

In April 2016, freshwater fishes of six species from Antsohihy (Anjingo River), Madagascar (14°53' S, 47°54' E) were examined

* – corresponding author

Fig. 1. **A** – *Ptychochromis* cf. *inornatus* from Madagascar, definitive host of *Schyzocotyle acheilognathi* (Yamaguti, 1934) **B** – Anterior part of *S. acheilognathi* with scolex.

for the presence of metazoan parasites. Among them, originally unidentified species of the endemic cichlid genus *Ptychochromis* Steindachner, 1880, designated as *Ptychochromis* sp. 1 (Perciformes: Cichlidae; field nos. 18/1; 19/2; 21/4; 22/5; 30/11; 32/13), was infected with 43 specimens of *S. acheilognathi* (prevalence 40 %; intensity of infection 1 – 18; mean intensity ± SD 7.5 ± 8.2; mean abundance ± SD 3.0 ± 6.2).

Tapeworms were fixed in 70 % ethanol under pressure (for morphological evaluation[1]) and in 96 % molecular-grade ethanol (for DNA sequencing). The specimens fixed with 70 % ethanol were stained with Mayer's carmine, dehydrated in an ethanol series, cleared with clove oil and mounted in Canada balsam; they were deposited as vouchers at the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS C-15/35).

Partial *cox*1 gene of one of the specimens was sequenced by J. Brabec using the method described by Brabec *et al.* (2016). We analyzed a segment (580 bp) of DNA from the mitochondrial large ribosomal subunit (16S) of RNA gene for our *Ptychochromis* sp. 1 using the same primers as applied for Malagasy and South African cichlids by Sparks and Smith (2004). The obtained DNA sequence was identical with the sequence of *Ptychochromis inornatus* from GenBank (accession number AY263876). Therefore, the host is designated as *Ptychochromis* cf. *inornatus*. The PCR conditions and methods followed Mendlová *et al*. (2012).

Results and Discussion

Tapeworms found in *Ptychochromis* cf. *inornatus* (Fig. 1A) belong to the family Bothriocephalidae because they possess median gonopores (a dorsally situated common cirro-vaginal pore and a ventral uterine pore) (Kuchta *et al*., 2008; Brabec *et al*., 2015). They were identified as *S. acheilognathi* based on the typical heart-shaped scolex bearing deep and narrow bothria with slit-like orifices (Fig. 1B; Pool & Chubb, 1985). Most tapeworms were not fully developed and only a few specimens contained gravid proglottids with the eggs in the uterus. Species identification was confirmed by the *cox*1 gene sequence, which corresponds to that of *S. acheilognathi* (J. Brabec – unpubl. data; see also Brabec *et al*., 2015, 2016).

None of the five other fish species examined including one native and endemic species, *Paretroplus lamenabe* Sparks, 2008 (Cichlidae), three native species, namely *Pachypanchax omalonotus* (Duméril, 1861) (Aplocheilidae), *Valamugil robustus* (Günther, 1861) (Mugilidae) and *Glossogobius giuris* (Hamilton, 1822) (Gobiidae), and one introduced species, *Oreochromis niloticus* (Linnaeus, 1758) (Cichlidae), were infected by this parasite.

Schyzocotyle acheilognathi has been reported from 9 countries of Africa including Morocco (new geographical and host records from *Carasobarbus fritchii* (Günther, 1874), *Luciobarbus massaensis* (Pellegrin, 1922), *L. rifensis* Doadrio, Casal-Lopez & Yahyaoui, 2015, *L. yahyaouii* Doadrio, Casal-López & Perea, 2016 and *L. rabatensis* Doadrio, Perea & Yahyaoui, 2015 – unpublished data of the present authors). However, no data on its occurrence in Madagascar are available (Gibson *et al*., 2005; Kuchta *et al*., 2012).

The origin and the way of the introduction of *S. acheilognathi* to this island remains unclear, even though a number of alien fish species have been introduced to Madagascar (Kiener, 1963). Many fish were introduced for aquaculture and others to improve fish production in natural water bodies. However, breeding fish often escaped from fishponds and succeeded well in wild habitats. The first two fish species, *Osphronemus gouramy* Lacépède and *Carassius auratus* (Linnaeus), were introduced to Madagascar from East Asia and Europe (France) in 1857 and 1861, respectively (Kiener, 1963). Common carp (*Cyprinus carpio* Linnaeus), which is one of the most susceptible definitive hosts of *S. acheilognathi*, was imported from France to Madagascar in 1916. From 1966

[1] – This fixation method, i.e. flattening of tapeworms and their fixation in 70 %, is not recommended; instead, heat-fixation, best with 4 % hot formaldehyde solution, should be used – see, e.g., Oros *et al.* (2010).

to 1972, juveniles of common carp were reintroduced into Lake Alaotra. In the 1950's and 1960's, several species of tilapias were also introduced (Kiener, 1963), but *S. acheilognathi* has never been reported from any of the introduced or native fish (Khalil & Polling, 1997; Gibson *et al*., 2005; R. Kuchta – unpubl. data).
Cichlids and perciform fishes in general are not the most common definitive hosts of *S. acheilognathi*. A total of 12 species of perciform fish (out of more than 200 fish species) were previously reported as hosts of *S. acheilognathi*, mostly from Mexico, with two records from *Oreochromis niloticus* (Linnaeus) in South Africa and Nigeria (Paperna, 1996; Ogbulie *et al*., 2011).

Conclusion

The present report of *S. acheilognathi* from Madagascar, which is a new geographical and host record of this invasive parasite, is considered to be of concern from the veterinary and conservation point of view, especially because this parasite was found in an endemic fish and with a high prevalence and intensity of infection. Even though the pathological effect of *S. acheilognathi* on cichlid fishes has not been studied, the observed infection rate indicates that this tapeworm has successfully colonized a new region, an isolated island in the Indian Ocean, most probably as a result of human activities. Therefore, inspection of cichlids and other fishes from the locality and surrounding water bodies is strongly recommended to detect possible spreading of this invasive and potentially dangerous fish parasite.

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Research Note

First report of the lesion nematodes: *Pratylenchus brachyurus* and *Pratylenchus delattrei* on tomato (*Solanum lycopersicum* L.) plants in Cape Verde

Ł. FLIS[1]*, R. DOBOSZ[2], K. RYBARCZYK-MYDŁOWSKA[1], B. WASILEWSKA-NASCIMENTO[3], M. KUBICZ[1], G. WINISZEWSKA[1]

[1]Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland, E-mail: *lflis@miiz.waw.pl*, *katarzynar@miiz.waw.pl*; *nicien@miz.waw.pl*; *mkubicz@miiz.waw.pl*; [2]Institute of Plant Protection-National Research Institute, Węgorka 20, 60-318, Poznań, Poland, E-mail: *r.dobosz@iorpib.poznan.pl*; [3]University of Cape Verde, Palmarejo, CP 279-Praia, Republic of Cape Verde, E-mail: *beata.nascimento@gmail.com*

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Summary

Roots of *Solanum lycopersicum* L. were collected in growing season of year 2015, on the island of Santiago in Cape Verde. Morphological, morphometric and molecular (18S rDNA and 28S rDNA) studies revealed the presence of *Pratylenchus brachyurus* and *P. delattrei* in root systems and root zones of tomato plants. To our knowledge, this is the first record of the occurrence of these nematode species in Cape Verde.
Keywords: Cape Verde; new geographic record; *Pratylenchus brachyurus*; *Pratylenchus delattrei*; 18S rDNA; 28S rDNA

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the vegetable crops most widely grown on irrigated land in the Republic of Cape Verde. Little is known about the plant-parasitic nematodes responsible for pest infestations of this economically important crop in this area. So far only *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Germani, 1978), *Heterodera schachtii* Schmidt, 1871 (Sturhan, 1993) as well as some representatives of the genus *Meloidogyne* Göldi, 1892 (Netscher & Taylor, 1976) and *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Flis *et al*., 2018) have been identified as nematodes parasitising tomato roots in Cape Verde. During the study of plant parasitic nematodes associated with tomato cultivation in Cape Verde, representatives of lesion nematodes *Pratylenchus* (Filipjev, 1936) were found. Morphometric analysis and molecular study allowed to identify the two investigated species as *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev et Schuurmans Stekhoven, 1941 and *P. delattrei* Luc, 1958. *Pratylenchus* spp. have significant economic impacts. Crop damage and yield losses by these nematodes have been previously observed (Egunjobi, 1974; Talwana *et al*., 2016). Those migratory endoparasites move within host root tissues causing necrosis and creating wounds, thus providing openings for soil-borne plant pathogens to enter and cause disease. In tropical and subtropical regions *Pratylenchus* spp. infect roots of several crops. *P. brachyurus* has been recorded parasitising pinapple roots in Hawaii (Godfrey, 1929), Uganda (Bafokuzara, 1982) and Brazil (Monteiro & Lordello, 1972); citrus and forest trees in North America (Brooks & Perry, 1967; Ruehle, 1971), coffee in Brazil (Lordello *et al.,* 1968), rubber in India (Mukherjee *et al.,* 2000) and many other species of economically important plants (Luc *et al.,* 2005; Castillo & Vovlas, 2007; De Araujo Filho *et al.,* 2014). *P. brachyurus* has also been reported from a few countries in Europe, in Bulgaria on tobacco (Katalan-Gateva & Nedechlev, 1983), on several crops in Russia (Ryss, 1988) and Australia (Riley & Kelly, 2002). *P. delattrei* primarily described from Madagascar (Luc, 1958) has also been found on several crops in Vietnam (Ryss, 1988), on sugarcane in Sudan (Saadabi, 1988), in ornamental plants, like rose and rhapis, grown in Korea (Kim & Minagawa, 1996), in date palm in Oman (Mani *et al.,* 2005) and in several hosts and localities in India (Jothi *et al*., 2004). In this article we provide morphological description and morphometric data of *P. brachyurus* and *P. dellatrei* from Cape

* – corresponding author

Table 1. Morphometrics of *Pratylenchus* spp. from tomato in Cape Verde. All measurements are in µm and in format: mean ± S.D. (range).

Character/ratios	*Pratylenchus brachyurus* (Godfrey 1929)	*Pratylenchus delattrei* Luc 1958
n	14	20
L	517 ± 45 (414 – 573)	532 ± 33 (498 – 586)
a	24.2 ± 1.9 (20.1 – 26.7)	26.6 ± 2.2 (22.1 – 31.3)
b	6.3 ± 0.5 (5.1 – 6.9)	6.6 ± 0.5 (6.1 – 7.7)
b'	3.7 ± 0.4 (2.9 – 4.1)	4.5 ± 0.4 (4.0 – 5.3)
c	20.1 ± 2.4 (15.3 – 25.5)	21.9 ± 2.1 (18.5 – 25.1)
c'	2.2 ± 0.2 (1.7 – 2.5)	2.2 ± 0.2 (1.9 – 2.8)
V	86 ± 1 (84 – 88)	76 ± 1 (75 – 78)
Stylet length	18.3 ± 0.4 (17.8 – 18.9)	16.4 ± 0.4 (15.4 – 16.9)
Dorsal gland opening	2.2 ± 0.3 (1.6 – 2.6)	2.9 ± 0.3 (2.4 – 3.1)
O	12.0 ± 1.9 (8.6 – 14.6)	17.4 ± 1.7 (14.3 – 21.3)
Pharynx length	82.1 ± 1.7 (79.2 – 85.2)	80.1 ± 3.2 (73.1 – 84.2)
Pharyngeal overlap	47.7 ± 7.9 (37.0 – 62.3)	39.0 ± 7.2 (29.8 – 49.0)
Maximal body diameter	21.5 ± 1.9 (17.7 – 26.0)	20.2 ± 2.2 (16.8 – 23.7)
Anal body diameter	12.2 ± 1.1 (15.9 – 20.9)	11.2 ± 1.2 (8.9 – 13.2)
Tail length	25.9 ± 1.9 (22.0 – 28.3)	24.8 ± 2.3 (21.0 – 27.1)
Tail annuli	18 ± 2 (16 – 23)	19 ± 2 (16 – 24)
Phasmid to terminus	14.3 ± 1.5 (11.6 – 16.9)	10.6 ± 2.7 (6.0 – 14.9)

Abbreviations are defined in Siddiqi (2000)

Verde. Genetic characteristics of LSU (28S rDNA) and SSU (18S rDNA) from analysed populations and results of comparative study of these sequences are also supported.

Material and Methods

Eight tomato plants with soil surrounded root systems were harvested in June 2015 in the village of Achada Colaço (located at 15°06'35.3"N, 23°31'31.9"W). Nematodes from roots were isolated by the modified Baermann method, whereas specimens from soil samples were extracted using the decanting and sieving method. Females assigned for morphological analysis were fixed in a triethanolamine formalin water solution (TAF) and mounted in anhydrous glycerine (Seinhorst, 1959) on slides using the paraffin-ring method. Morphological observations and morphometric analyses were performed using a Leica light microscope with the Nomarski differential interference contrast. Females selected for the genetic analysis (three specimens for each species) were fixed in a DESS solution (Yoder *et al.,* 2006). After washing in sterilized milli-Q water, single nematode individuals were used for DNA extraction according to the nematode lysis procedure, as described by Holterman *et al*. (2006). 18S rDNA gene fragments were amplified in PCR reactions with the aid of 988F and 1912R, as well as 1813F and 2646R primer combinations (Holterman *et al.,* 2006). Amplification of the partial 28S rDNA sequence was obtained using primers 61F (Holterman *et al.,* 2008) and MCB1R (Dobosz *et al.,* 2013). The 18S and 28S rDNA regions were sequenced by the Sanger method on ABI 3500L genetic analyzer (Applied Biosystems, Foster City, CA, USA). The newly obtained 28S rDNA sequences were implemented in a phylogenetic analysis concerning relationships within clades IV ('*P. penetrans* group') and VI ('*P. zeae* group') of the genus *Pratylenchus* as defined by Subbotin *et al.* (2008) and Palomares-Rius *et al.* (2014). Initial multiple sequence alignment was performed using the BioEdit program (v. 7.2.5; Hall, 1999) and included publically available (GenBank) representatives of '*P. penetrans* and *P. zeae* groups'. All available unique *P. brachyurus* and *P. delattrei* sequences were used. In order to root the tree two outgroup sequences (FN433867 and JN967754) were chosen based on the total nematode phylogeny by Van Megen *et al.* (2009). The final multiple-sequence alignment contained 663 positions. Substitution models were tested using "FindModel", an online implementation of the MODELTEST program (Posada & Crandall, 1998). The General Time Reversible plus Gamma substitution model was selected. The Bayesian phylogeny was constructed with the program MrBayes (v. 3.1; Ronquist & Huelsenbeck, 2003). Four independent runs were performed with four Markov chains per run. The program was run for 800,000 generations with a sample frequency of 200 generations. The sampled trees from each run were combined in a single 50 % majority-rule tree. Stabilisation of the likelihood and parameters was checked with the program Tracer (v. 1.6; Rambaut *et al.*, 2014).

Results and Discussion

Morphological and morphometric analyses of the investigated females confirmed their affiliation to *P. brachyurus* and *P. delattrei*. The morphology of *P. brachyurus* from Cape Verde (14 females) was characterised by: low and angular lip region with two

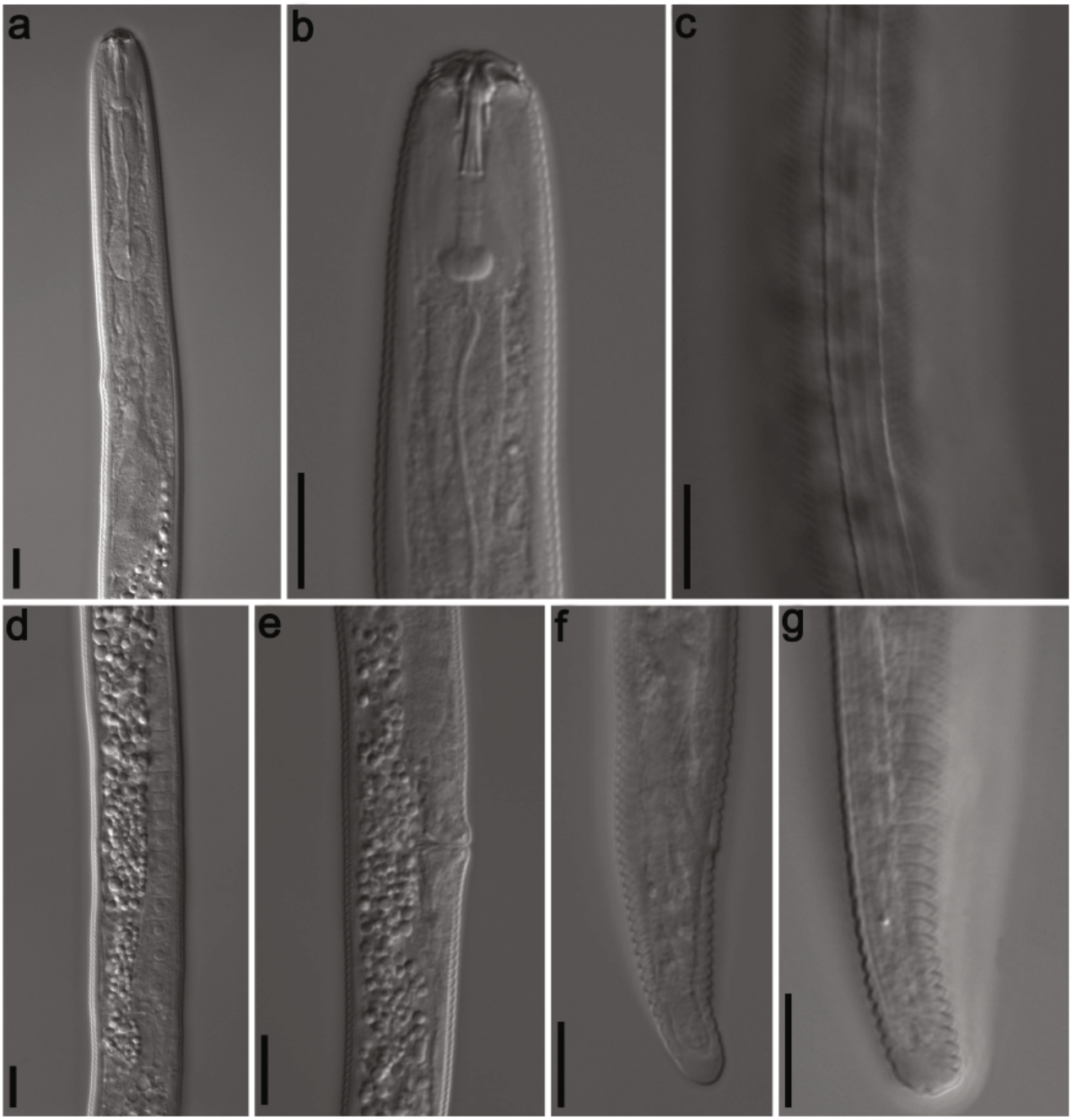

Fig. 1. *Pratylenchus brachyurus* (Godfrey 1929) female from Cape Verde. **a.** pharyngeal region; **b.** anterior end; **c.** lateral field at mid-body; **d.** ovary with single row of oocytes; **e.** vulval region; **f.** tail; **g.** tail, phasmid. (All scale bars = 10 µm)

lips annuli, which is separated from body contour; long spear with rounded, laterally directed knobs; lateral field with four lines; a single row of oocytes; empty and rounded spermatheca; posterior vulva; undifferentiated and well developed postvulval uterine sac; conoid tail with rounded (to truncate), smooth or broadly annulated terminus. The obtained results agreed with the previous descriptions of *P. brachyurus* (Loof, 1978; Castillo & Vovlas, 2007) (Fig. 1, Table 1). The morphology of *P. delattrei* from Cape Verde (20 females) was characterised by continuous body contour, more or less truncate lip region with three annuli; medium spear, with rounded and slightly anteriorly directed knobs; lateral field with four lines; two rows of oocytes; empty and rounded spermatheca, posterior vulva; undifferentiated and well developed postvulval uterine sac; subcylindrical tail with rounded and smooth terminus

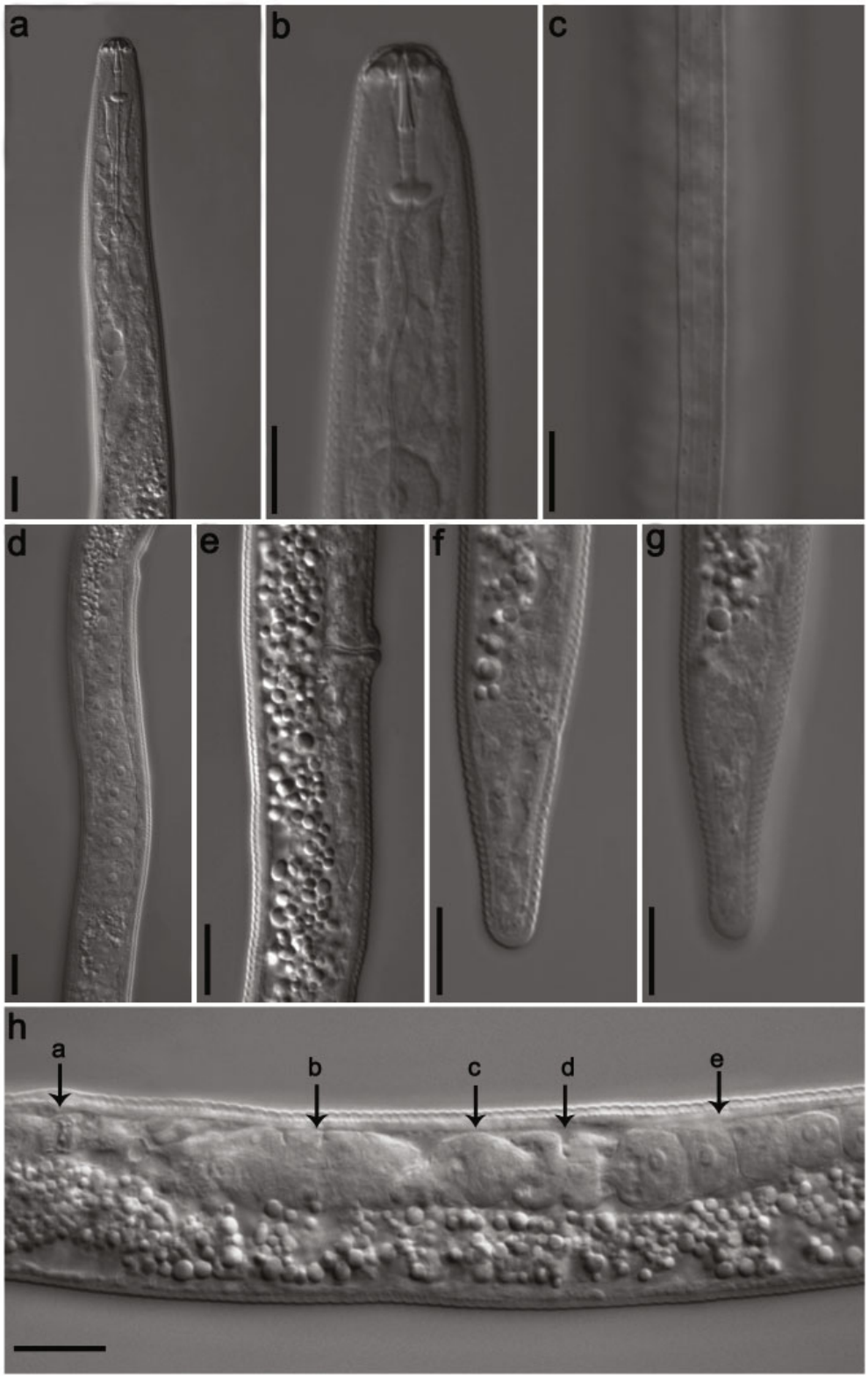

Fig. 2. *Pratylenchus delattrei* Luc 1958 female from Cape Verde. **a.** pharyngeal region; **b.** anterior end; **c.** lateral field at mid-body; **d.** ovary with double rows of oocytes; **e.** vulval region; **f.** tail; **g.** tail, phasmid; **h.** genital system: a - vulva, b - crustaformeria, c - spermatheca, d - oviduct, e - ovary. (All scale bars = 10 µm)

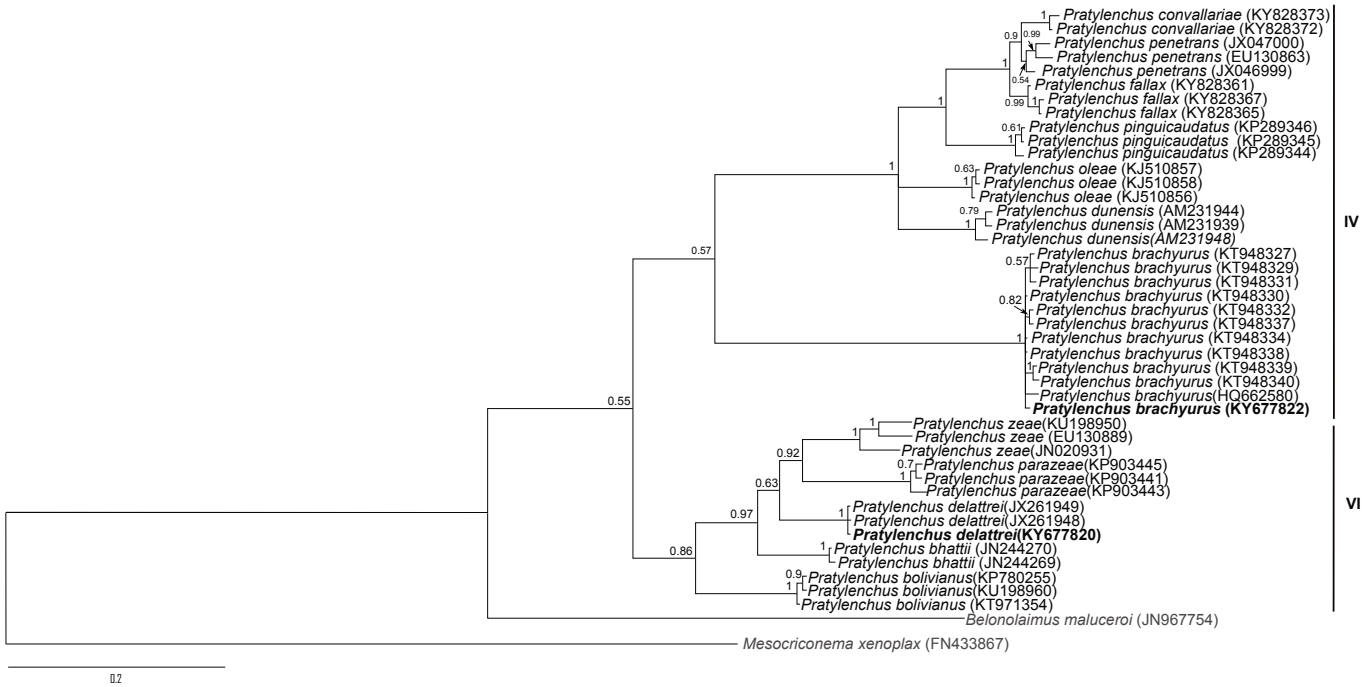

Fig. 3. *Pratylenchus* spp., 28S rDNA-based Bayesian phylogenetic tree revealing relationships within clades IV ('*P. penetrans* group') and VI ('*P. zeae* group'). Numbers near nodes stand for posterior probabilities. The newly obtained 28S rDNA sequence of *P. brachyurus* and *P. delattrei* from Cape Verde are indicated in bold.

(Fig. 2, Table 1). Morphological characteristics of *P. delattrei* from Cape Verde are in line with descriptions of previously investigated materials (Luc, 1958; Loof, 1978; Castillo & Vovlas, 2007; Majd Taheri *et al*., 2013). A comparison of morphometric analysis findings between the specimens concerned and those previously described showed cross-population quantitative differences in tail annulation in females. Studies so far have indicated that the numbers of tail annuli in *P. delattrei* females varies from 17 to 25. According to Ryss (1988) and Loof (1978) the number of annuli does not exceed 20. However, our analysis as well that by Bahmani *et al.* (2013) and Majd Taheri *et al.* (2013) has pointed out the occurrence of specimens exhibiting an even higher number of tail annuli.

The amplification of the almost full-length 18S rDNA fragment was successful only in the case of *P. delattrei* (1672 bp, KY677819) while for *P. brachyurus* we were able to amplify only the second part of the 18S rDNA gene (810 bp, KY677821). To our knowledge this is the first report of a 18S rDNA sequence of *P. delattrei* while the GenBank available 18S rDNA partial sequences of *P. brachyurus* concern the first part of the gene (EU130795-EU130797, EU13084). Therefore, it was not possible to align the GenBank derived 18S rDNA sequences with the second part of the 18S rDNA gene partial sequences, obtained from the *P. brachyurus* from Cape Verde. The Basic Local Alignment Search for 28S rDNA from *P. brachyurus* (521bp) (KY677822) showed a 98 % similarity to the sequences of *P. brachyurus* deposited in GenBank from Brazil (KT948327, KT948329-KT948332, KT948334, KT948337-KT948340, HQ662580). The 28S rDNA sequence alignment from *P. delattrei* (718 bp) (KY677820) showed a 99 % similarity to another sequence of *P. delattrei* deposited in the GenBank from Iran (JX261948, JX261949). As expected in the resulted Bayesian tree (Fig. 3), the newly acquired sequences from Cape Verde had been positioned together with corresponding, GenBank available *P. brachyurus* and *P. delattrei* representatives. As observed in previous works by Palomares-Rius *et al.* (2014) and Janssen *et al.* (2017) *P. brachyurus* belongs to *'P. penetrans* group' and has been localised as a separate, basal branch in the clade IV. *P. delattrei* has been positioned in clade VI in close relationships to *P. parazeae*, *P. zeae*, *P. bhatti* and *P. bolivianus*.

Representatives of *P. brachyurus* and *P. delattrei* species are considered economically important plant-parasitic nematodes that are widespread mainly in tropical environments (Godfrey, 1929; Loof, 1978; Castillo & Vovlas, 2007). It is noteworthy that the distribution range of *P. brachyurus* is broader. This species has been detected on all continents except Antarctica (Tarjan & O'Bannon, 1969; Castillo & Vovlas, 2007). This report broadens our knowledge of nematode biodiversity of Cape Verde as well of the morphometry and molecular characteristics of two economically important species *P. brachyurus* and *P. delattrei*.

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