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Morphological observations and the effects of artificial digestive fluids on the survival of *Diploscapter coronata* from a Japanese patient

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Abstract

Unusual non-human parasitic nematodes and eggs were detected in the faeces of an 8-year-old Japanese female suffering from Henoch-Schönlein purpura. The worms were adult female rhabditiform nematodes measuring 325.6–441.2 μm in length and 18.3–26.5 μm in width. One pair of the labia oris was notched with many spiny projections, while the other pair was strongly curved outwards. The worms were identified using light and scanning electron microscopy as the free-living nematode *Diploscapter coronata* (Cobb) based on their characteristic morphology. The patient's faeces containing worms and eggs were cultured using a filter-paper culture technique and after 7 days of culture, male as well as female worms were recovered. Worm survival time and hatchability of the eggs were examined *in vitro* after treatment with an artificial gastric or intestinal fluid. Although adult worms survived for less than one minute, eggs hatched after treatment with artificial gastric fluid. This suggests that eggs accidentally ingested or produced by adult *D. coronata* could develop in the human gastro-intestinal tract. Some morphological features of male *D. coronata* are also described.

Introduction

Diploscapter coronata (Cobb) (Rhabditidae) is a free-living nematode found in soil in warm countries as well as in the roots of diseased plants (Cobb, 1913). To date, only facultative cases have been reported in humans, and the worms are assumed to have been acquired from decaying vegetation (Beaver *et al.*, 1984). Chandler (1938) reviewed nine human cases of stomach infections involving this organism. All of these patients suffered from achlorhydria, and the author doubted that *D. coronata* infections were generally established in the human intestine. Several cases of urinary infection have been reported in Israel (Witenberg, 1951; Beaver *et al.*, 1984) and Taiwan (Yokogawa, 1936). In the latter case, a 72-year-old Japanese woman was admitted to hospital with acute pyelitis, and nematodes were only identified in the urinary sediment of the patient when the urine showed an alkaline reaction. Therefore, most cases are considered to be transitory and accidental infections.

In the present study, *D. coronata* were detected in the faeces of an 8-year-old female suffering from Henoch-Schönlein purpura (HSP), and morphological

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features of the worms and eggs are described using light and scanning electron microscopy (SEM). Furthermore, the influence of artificial digestive fluids on the hatchability of the eggs and survival of adult worms *in vitro* are investigated, and the infectivity of this species to humans and its relationship with HSP are discussed.

Materials and methods

Case report

The patient was an 8-year-old female living in Kochi Prefecture, Japan, with no history of overseas travel. She had occasionally complained of bilateral knee arthralgia during the last 10 days of February, 2002. At that time, a purpura was recognized on the extensor side of the crura, but recovery occurred without any treatment. She had common cold symptoms from 1 March 2002. During the night of 12 March, she had pain in the left ear and the abdomen, together with petechiae and purpura of the legs. On 13 March, an otolaryngologist diagnosed left acute otitis media, performed a myringotomy and gave her an antibiotic (cefcapene pivoxil HCl; CFPN-PI). The patient saw a dermatologist on 14 March, since the purpura had also appeared on the upper limbs. She was diagnosed as HSP, given dexamethasone ($1\,mg\,day^{-1}$) and admitted to Kochi Medical School Hospital on 15 March. Laboratory data and the clinical course of the patient after hospitalization are shown in table 1. On 15 March, laboratory data revealed a white blood cell count of $9300\,mm^{-3}$, red blood cell count of $4{,}770{,}000\,mm^{-3}$, haemoglobin level of $13.7\,g\,dl^{-1}$, eosinophil count of 1.0%, platelet count of $425{,}000\,mm^{-3}$, C-reactive protein level of $0.4\,mg\,dl^{-1}$ and an RA-test result of $1+$. In urinalysis, her protein concentration was $10\,mg\,dl^{-1}$ and occult blood was positive (grade 1). Blood chemistry and coagulation examinations were within normal limits. Faecal occult blood was positive (grade 1), but haemoglobin was negative. The administration of carbazochrome sodium sulphonate was initiated on 22 March, as the patient frequently complained of abdominal pain after hospitalization. A purpura appeared over a wide area involving the legs, abdomen and buttocks. There were no findings indicating the presence of arthritis. The patient complained of mild abdominal tenderness, but the pain was within the self-control range. After 23 March, the complaint of abdominal pain disappeared and the purpura gradually went into remission. The patient showed a tendency toward constipation. A coprostasis was recognized by abdominal radiography. In a skin biopsy, neutrophils and lymphocytes were found to have infiltrated the circumvascular regions, but deposits of immunoglobulin (Ig)G, IgA, IgM, C3, C4 and C1q were not observed using a direct fluorescently-labelled antibody method. Parasitological examinations were carried out on a fresh faecal sample. In a direct faeces specimen examination, many motile nematodes and some eggs were observed by light microscopy on 25 March. However, no worms or eggs were detected in faeces collected from the patient on 27 and 28 March. On 1 April 2002, the patient left hospital because her symptoms had reduced.

Faecal cultures

Eggs, larvae and adults were obtained from the patient's faeces using a filter-paper culture method (Harada & Mori, 1951). Worms were cultured and maintained by the filter-paper culture technique using parasite-free human or murine faeces. Faecal samples including eggs and worms were spread on one side of the filter paper strip ($1 \times 8\,cm$) (Advantec no. 50, Toyo Roshi, Tokyo, Japan). The filter paper was placed in a test tube containing 5 ml of distilled water and incubated at room temperature (23–25 °C) for seven days. Emerged worms were collected and/or inoculated in a new culture.

Morphology

Worms and eggs were observed by light microscopy and SEM. The specimens for SEM were processed as follows. After collection from the culture fluid using the Army Medical School (AMS III) method and Sheather sucrose flotation (Sheather, 1923), worms were washed with PBS (pH 6.8) and killed by heating in a water bath at 56 °C for 1–2 min. Worms were then fixed with 10% formalin for 2 h at room temperature, rinsed three times in PBS, post-fixed in 2% OsO_4 for 1 h, rinsed in distilled water and finally dehydrated at 15 min intervals through a graded ethanol series and isoamyl acetate. Next, the worms were critical point dried in liquid CO_2, mounted on stubs, coated with gold and viewed under an Hitachi S-2380N SEM.

Culture conditions

The survival time of adult worms and hatchability of eggs were tested in artificial digestive fluids, namely 1% hydrochloric acid–1% pepsin aqueous solution (pH 1.0) and 1% trypsin–Hanks' balanced salt solution (HBSS) (pH 6.8) (Nissui, Tokyo, Japan). A total of 50–80 worms were cultured in serial dilutions of the digestive fluids ($100\,\mu l$ per well). The survival time was determined as the duration of time required until worm motility had completely stopped. Eggs were collected using a pipette after each faecal sample had been dissolved with 5 ml distilled water and filtered with gauze. Approximately 200 eggs were pre-incubated in a $100\,\mu l$ per well of artificial gastric fluid for 2 h at room temperature, and incubated in HBSS (pH 6.8) after washing with distilled water. The influence of temperature on the survival rate of adult worms was examined. A total of approximately 355 worms per tube were cultured with faeces from healthy subjects at 23, 28 and 37 °C. The numbers of motile worms were counted after 1, 3 and 7 days of culture.

Results

Morphology

A total of 267 worms (adults and larvae) as well as eggs at various developmental stages were collected from a

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Table 1. Clinical course of an 8-year-old female Japanese patient, suffering from Henoch-Schönlein purpura and infected with *Diploscapter coronata.*

	Days (2002)								
	3/15	3/18	3/19	3/21	3/22	3/23	3/25	3/27	3/28
Peripheral blood cell count									
RBC ($\times 10^4\,mm^{-3}$)	477						487		449
Hb(g dl^{-1})	13.7						14.1		12.9
WBC ($\times 10^4\,mm^{-3}$)	9.3						9.0		5.9
Plt ($\times 10^4\,mm^{-3}$)	42.5						44.5		47.4
Serological test									
CRP (mg dl^{-1})	0.4						1.0		0.1
IgG (mg dl^{-1})	844								
IgA (mg dl^{-1})	262								
IgD (mg dl^{-1})	<1								
IgM (mg dl^{-1})	136								
RA-test	Positive								
ASLO	<30								
ASK	<40								
HPV B19 IgM/IgG	0.63/0.21								
MP-CF	<4								
Coagulation test									
PT (sec(%))	11.1(94.2)								
APTT (sec)	33.5								
F-13 (%)	89								
Faecal examination									
Occult blood/haemoglobin		1 + /−	1 + /+ −				1 + /+		− /−
Parasitological examination							+[a]	−	−
Body temperature (°C)	36.3	36.0	36.4	36.2	36.9	36.5	36.6	36.2	36.2
Symptoms									
Abdominal pain	−	−	+	+	+	+	−	−	−
Purpura[b]	N	N	N	=	N	=	=	=	−
Defecation (per day)									
Medicine	Cefcapene pivoxil HCl (CFPN-PI) Dexamethasone Marzulene-S				Carbazochrome sodium sulfonate				
Meal	Feeding in hospital								

[a]D. Coronata detected in the faeces.
[b]New purple spot (N); no change(=); disappearance (−).

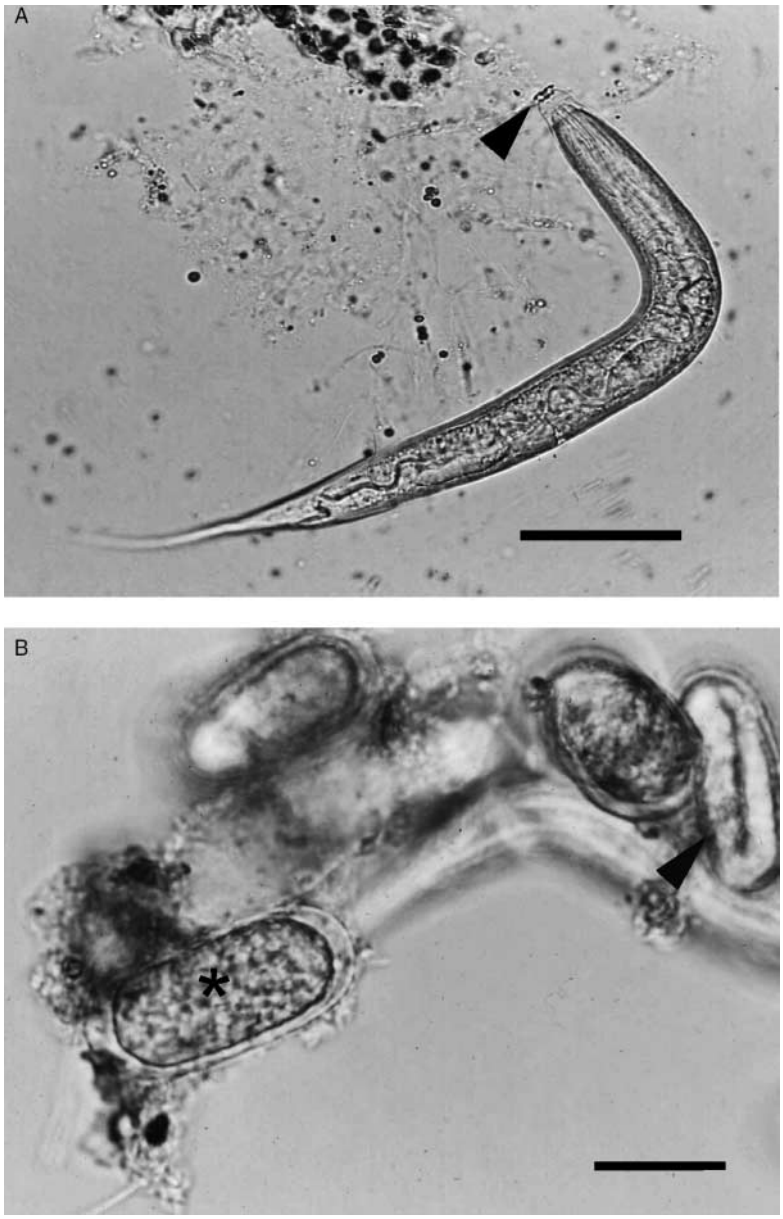

Fig. 1. Adult worm (A) and eggs (B) of *Diploscapter coronata* observed in a direct smear of a faecal specimen from an 8-year-old female patient. A, Lips of two pairs of hooks at the cephalic end of an adult worm (arrowed); B, eggs including a cell (asterisk) and a larva (arrowed). Scale bars 50 μm (A), 20 μm (B).

sample of the patient's faeces. Some worms and eggs were fixed for microscopical examination (fig. 1A and B), whilst the remainder were used for filter-paper cultures. No differences were observed in the morphology of the specimens from the faecal sample and those from the filter-paper cultures.

Adult female worms are 325.6–441.2 μm long and 18.3–26.5 μm wide, with an oesophageal bulb of 68.0–85.5 μm in length and an oesophageal endbulb of 15.4–20.1 μm in length (fig. 2A). At the cephalic end, there are two pairs of lips. The dorsal and ventral lips are modified into strong outwardly-curved hooks with two points (probably representing fused lips), while the lateral lips extend forwards. One pair of the labia oris is notched with many spiny projections (fig. 2B and C), while the other pair is strongly curved outwards with a forked tip (fig. 2D). The vulva, which opens in the mid region of the body, has a pair of opposed reflexed ovaries. Two lateral lines on the cuticular surface of the body were observed in both adult males and females by SEM (fig. 2E). The female tail is conical (fig. 2F). Eggs are 44.3–48.7 μm long and 20.1–21.9 μm wide (fig. 2G).

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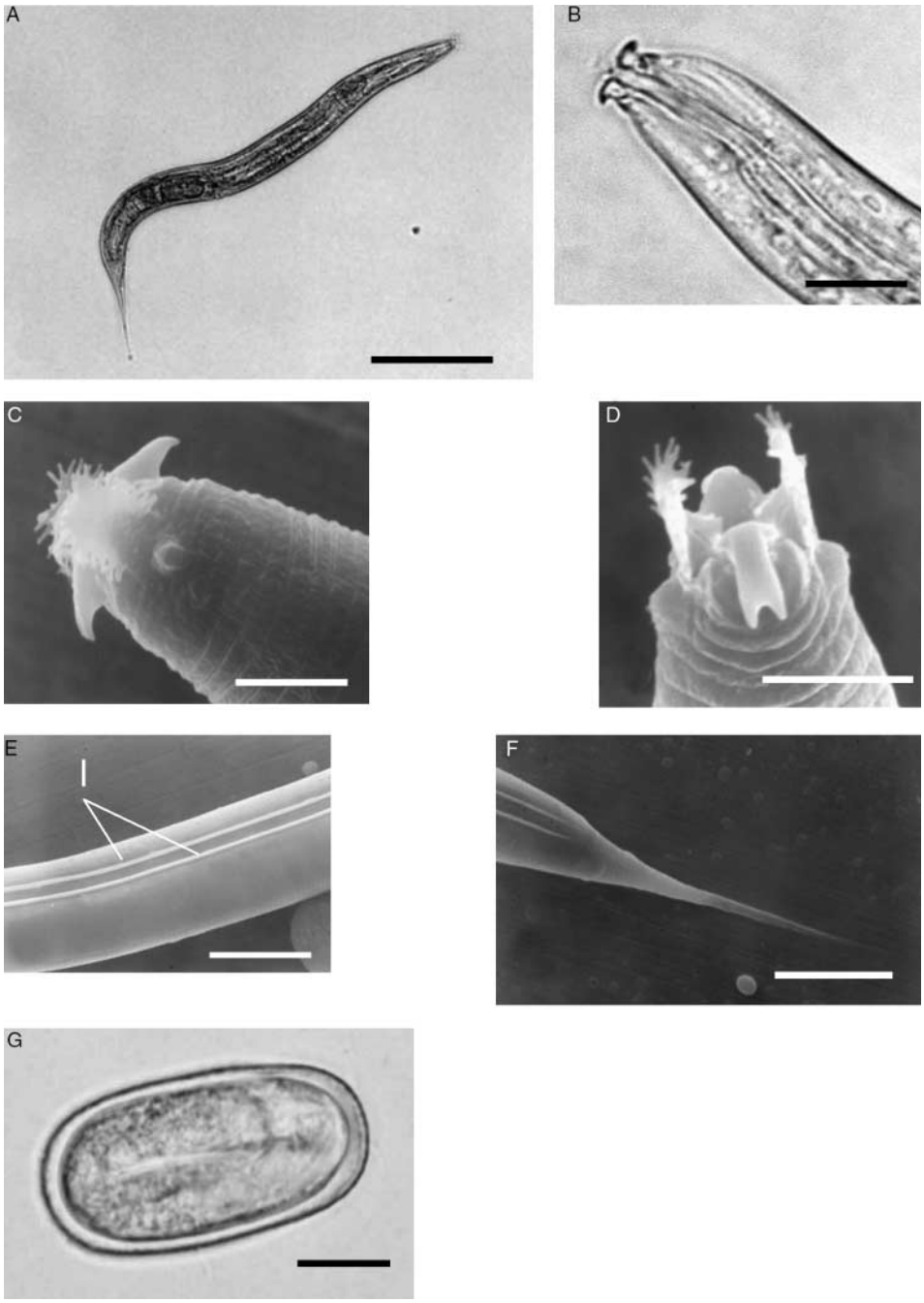

Fig. 2. Morphology of female *Diploscapter coronata*. A, adult; B–D, mouth and lips; E, lateral lines (l) on the cuticular surface; F, tail; G, egg. Scale bars 100 μm (A), 10 μm (B, G), 5 μm (C, D), 20 μm (E, F).

Adult males are 348.4–393.6 μm long and 22.3–27.8 μm wide, with an oesophageal bulb of 70.1–72.0 μm in length and an oesophageal endbulb of 16.9–19.5 μm in length (fig. 3A). The stoma is long and narrow, and the oesophagus is rhabditoid (fig. 3B). A muscular pharynx (oesophagus) is present with a corpus leading to the middle pseudobulb and connected to the posterior endbulb by a narrow isthmus. The male tail bears caudal alae. The two spicules are 22.3–26.2 μm in length (fig. 3C) and brownish with knobbed ends and an uncinate tip. The gubernacula are 10.9–13.4 μm long and are located under the tips of the spicules (fig. 3D). There are thin-walled extensions on the postero-lateral body margins with six pairs of genital papillae, and finger-like projections on the ventral surface (fig. 3E and F).

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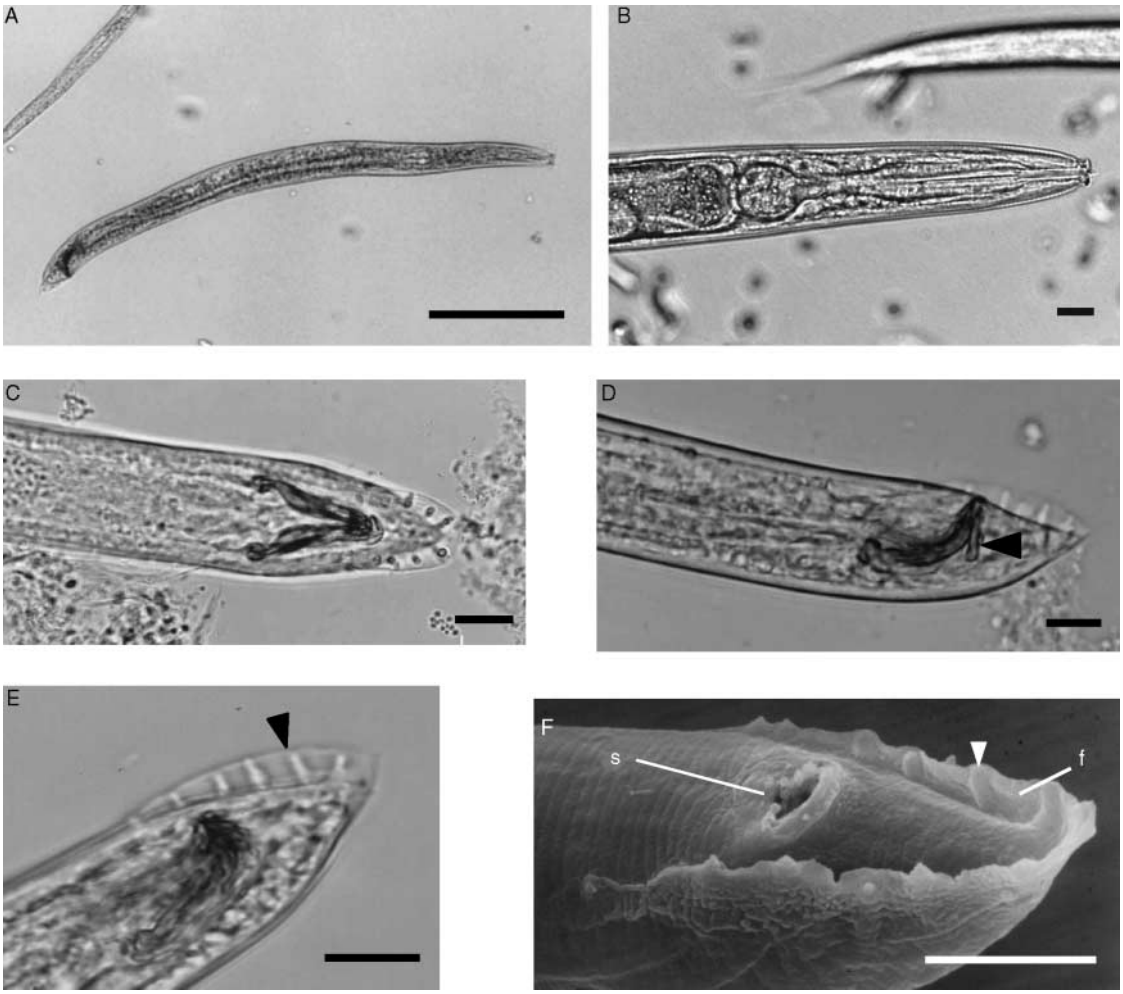

Fig. 3. Morphology of male *Diploscapter coronata*. A, adult; B, oesophagus (rhabditoid form); C, two spicules on the tail; D, gubernacula (arrowed) under the tips of the spicules on the ventral surface; E, six pairs (arrowed) of genital papillae and finger-like projections on the ventral surface; F, tail of a male showing caudal alae and rays (f) as well as the anus and spicules (s) and six pairs of genital papillae (arrowed). Scale bars 100 μm (A), 10 μm (B–F).

Based on these morphological features, we identified the nematode as *Diploscapter coronata* (Cobb, 1893, 1913).

Worm survival time and egg hatching in artificial digestive fluids

Although adult worms were able to survive in artificial intestinal fluid for at least 6 to 20 h (data not shown), their survival time was less than 1 min in artificial gastric fluid (table 2). Furthermore, the adult survival time corresponded to serial dilutions of the artificial digestive fluid. Larvae were able to hatch from eggs treated with artificial gastric fluid (table 3), but the hatchability of eggs treated with high concentrations of artificial gastric fluid was significantly lower than that of control eggs. The influence of temperature on survival rate of adult worms

Table 2. Effects of various concentrations of artificial gastric fluid on the survival of adult *Diploscapter coronata*.

Dilutions of artificial gastric fluid	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
pH	1.0	1.1	1.3	1.5	1.9	2.2	2.5	2.8
Survival time (min)	0.9 ± 0.1	2.3 ± 0.1	6.5 ± 0.9	15.0 ± 2.8	33.6 ± 0.3	55.0 ± 3.0	62.0 ± 4.0	73.4 ± 7.0

Values represent the mean ± SEM ($n = 3$).

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Table 3. Hatching of *Diploscapter coronata* eggs treated with artificial gastric fluid.

Dilutions of artificial gastric fluid	Culture time (hours)			
	24	48	72	96
1/2	2.6 ± 1.7	3.4 ± 2.5	0.6 ± 0.3**	0.1 ± 0.1
1/8	3.4 ± 1.6	5.4 ± 0.3	2.6 ± 0.1**	0.4 ± 0.2
1/32	6.9 ± 4.1	10.6 ± 2.2	5.5 ± 0.7*	1.8 ± 1.1
1/128	10.4 ± 6.5	14.3 ± 1.7	11.2 ± 0.8	3.1 ± 1.1
Control	7.0 ± 3.5	12.3 ± 1.8	10.3 ± 0.7	3.8 ± 0.5

Values represent the mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.001$, vs. the control.

was tested. During 7 days of observation, both 23 and 28 °C were found to be the most suitable for worm survival, whereas 37 °C was unsuitable (table 4).

Discussion

Free-living nematodes were detected in the faeces of an 8-year-old female suffering from HSP. The worms were identified as *D. coronata* using the following characteristic features: they are rhabditiform, possessing an oesophagus with a terminal bulb and two pairs of lips; dorsal and ventral lips are modified into strong outwardly-curved hooks with two points, while the lateral lips extend forwards; one pair of labia oris is notched with many spiny projections and the other pair strongly curved outwards. The morphological features of the mouth also coincide with the keys of Anderson & Bain (1982) and Maupas (1900).

The males of *D. coronata* were previously described by Maupas (1900) who found 5–6 males per 1000 worms, and noted that all 150 immature worms developed into females laying fertile eggs, which developed normally. In the present study, male worms were detected using a faecal filter-paper culture technique and a male sex ratio of about 1% was found. Maupas (1900) reported that male worms are sexually impotent and described their morphology as having seven pairs of genital papillae. However, in the present study, six pairs of papillae with a gubenaculum near each spicule, were observed in male worms by light microscopy and SEM.

Diploscapter coronata was previously reported to survive in the host's gastric contents for about 48 h at room temperature, although worms died after overnight incubation at 37 °C (Chandler, 1938). In the present study, the survival time of adult worms in artificial gastric fluid depended on the pH of the culture. Therefore, it is assumed that orally ingested *D. coronata* cannot survive in healthy humans, but may survive in patients with achlorhydria or hypoacidity. It is unknown whether the patient in the present study was suffering from achlorhydria or hypoacidity. In contrast, larvae were able to hatch from eggs treated with artificial gastric fluid (table 3), although the acid level affected the hatchability. Furthermore, worms not only survived following *in vitro* culture in artificial intestinal fluid but also multiplied in faeces from normal subjects at 23, 28 and 37 °C. This suggests that *D. coronata* can survive in the human intestine after hatching from ingested eggs. This also supports previous findings that worms multiply rapidly in alkaline rather than acid urine (Yokogawa, 1936).

Table 4. Effects of temperature on filter-paper cultures of *Diploscapter coronata.*

Days	Temperature (°C)		
	23	28	37
0	355.0 ± 13.1	355.0 ± 13.1	355.0 ± 13.1
1	686.7 ± 64.2	766.7 ± 39.3	379.8 ± 72.1
3	862.5 ± 51.4	919.3 ± 76.2	166.7 ± 54.6
7	975.0 ± 79.7	1050.7 ± 113.5	0.0 ± 0.0*

Values represent the mean ± SEM ($n = 3$). *$P < 0.05$ vs. 23 °C.

It is highly likely that the patient in the present case ingested *D. coronata* eggs or adult females accidentally. The patient complained of abdominal pain and constipation, but it was unclear whether the worms caused these symptoms. The main clinical manifestations of HSP are palpable purpura, arthritis, abdominal pain, gastrointestinal bleeding and nephritis. Interestingly, the purpura and abdominal pain improved after the disappearance of *D. coronata.* In general, HSP attacks patients in the autumn and winter, frequently following an upper respiratory infection. Various causative agents are thought to trigger the disease, including infection with adenovirus, hepatitis B virus, coxsackie virus, herpes simplex virus, human immunodeficiency virus, human parvovirus B19, varicella virus, mycoplasma, *Helicobacter pylori*, *Streptococcus pyogenes* and *Toxocara canis* as well as drugs and environmental factors (Rai *et al.*, 1999; Saulsbury, 1999). In the present case, we could not exclude the possibility that *D. coronata* infection may have been involved in the development and clinical relapse of HSP.

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