

Effect of isolation method of *Contracaecum rudolphii* Hartwich, 1964 (Ascaridida: Anisakidae) eggs on embryonic development and the number of larvae hatched

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ABSTRACT. This study tested the isolation of *C. rudolphii* eggs using various methods and evaluated their course of embryogenesis and the number of larvae hatched. The pressing of eggs from the earlier-isolated uteruses was conducted in Petri dishes using bent preparatory needles to obtain eggs for a control culture. The experimental cultures contained incubated mature females in a culture medium which had been homogenized and digested by proteases. In the first experimental cultures, the females were incubated for three days at 40°C in 10-ml plastic flasks. Eage's medium with the addition of 1% pepsin (pH 2) and 20% heat-inactivated foetal bovine serum was used as an incubation fluid. In the second method, the suspension of females homogenized with a blender was centrifuged for 3 minutes at 1000 rpm, the supernatant was removed and the sediment was then rinsed with a PS. In the third method, an attempt was undertaken to collect eggs by digestion of mature females with 0.1% and 1% aqueous solutions of pepsin adjusted to pH 2.0 with 1N HCl, as well as in 0.1% and 1% solutions of trypsin prepared in a Sørensen buffer (pH 7.6). The suspension obtained after complete digestion and still containing eggs was purified from proteases by washing several times with PS. In turn, no eggs were isolated by using the method of incubation of females in a culture medium nor by digestion with pepsin. The method of homogenization of whole nematodes resulted in egg damage. The best method of egg isolation was digestion with a 0.1% solution of trypsin. When the digestion was conducted with a 1% trypsin solution, the arrestment of embryogenesis was observed in a considerable percentage of the eggs, whereas eggs thecae were left by 31% of the larvae.

Key words: Nematoda, *C. rudolphii*, eggs, embryogenesis

Introduction

Cultures of parasites *in vitro* are a very valuable research method applied in parasitology. They enable the elucidation of a number of issues referring to the physiology, development and function of a parasite–host system. They are also of special significance in immunological and antiparasitic drug examinations. Conducting a culture of larvae or mature individuals requires assuring conditions similar to those the parasite has in an intermediate or final host. To this end, an appropriate nutrient-rich culture medium as well as an appropriate temperature and gaseous phase should be provided. The cultured larvae may be isolated from tissues of intermediate hosts or obtained by hatching or by release from eggs under artificial conditions often with the use of

an additional mechanical or physicochemical stimulation [1–3]. The collection of eggs from mature females may proceed with various methods, which are likely to affect the embryonic development and the number of larvae hatched.

The most physiological method of egg isolation from nematodes, protecting against contact with the allergizing fluid of the body cavity, is *in vitro* culture of mature females. This method was also applied for the production of eggs of *Ascaris suum*, *Hysterothylacium aduncum* and *Anisakis simplex* [4–8].

A very rapid method of egg isolation is the homogenization of whole mature females or – in the case of larger nematodes – of previously-isolated uteruses [9,10]. Højgaard [11] was the first to apply this method to isolate the so-called “thin-shell” eggs of *A. simplex*.

A more labour-consuming method which poses a risk of contact with a strongly allergizing fluid filling the body cavity, is the mechanical pressing of eggs from whole mature females or from the terminal sections of earlier-isolated uteruses. In the case of small nematodes, it is not possible and produces large amounts of unwanted tissue detritus. This method is very commonly applied in the case of such nematodes as: *A. suum* [12], *Ascaridia galli* [13] *Syncuaria squamata* [14], and *Pseudoterranova decipiens* [15]. In addition, it was the only method used to isolate eggs from nematode *Contraecaecum rudolphii* [16–19].

The objective of the reported research was to identify the possibilities of isolating eggs of *C. rudolphii* with various methods and to determine the effect of those methods on the embryogenesis and number of hatched larvae.

Material and methods

Mature females of *C. rudolphii* were isolated from stomachs of cormorants shot in the area of Lake Selment Wielki and transported to a laboratory in thermos flasks with warm physiological saline (PS) with the addition of intestinal digesta of the cormorants. In the laboratory, the females were rinsed several times with PS containing 100 U penicillin/ml (Polfa, Tarchomin), 100 ml streptomycin/ml (Polfa, Tarchomin) and 100 U nystatin/ml (Pliva RX). Eggs were isolated from the mature females with the following methods: pressing the previously-isolated uteruses, incubation of nematodes in a culture medium and homogenization. In addition, an attempt was undertaken to obtain eggs by means of subjecting whole mature females to digestion with pepsin and trypsin. Five females with similar lengths were used in each method, and analyses employing the methods of females incubation and digestion were conducted in triplicate. The pressing of eggs from the earlier-isolated uteruses was conducted on Petri dishes with the use of a bent preparatory needles, and the eggs obtained in this way served as a control culture. In the second method, the females were incubated for three days at 40°C in 10-ml plastic flasks. Eagle's medium (BIOMED, Lublin) with the addition of 1% pepsin (1:100, Zakład Enzymów i Peptonów, Łódź) (pH 2) and 20% heat-inactivated foetal bovine serum (BIOMED, Lublin) was used as an incubation fluid. The presence of eggs on the bottom of a flask was checked every day. In the third method, the suspension of females homogenized

with a blender (Pol-Eko-Aparatura H 500, 10 000 rpm for 2 minutes) was centrifuged for 3 minutes at 1000 rpm (MPW-340) and the supernatant was then removed and the sediment was rinsed with a PS. The procedure was repeated 3 times. In the fourth method, an attempt was undertaken to collect eggs by digestion of mature females with proteases. Finally, the females were incubated at 37°C in 0.1% and 1% aqueous solutions of pepsin (1:100, Zakład Enzymów i Peptonów, Łódź) adjusted to pH 2.0 with 1N HCl, as well as in 0.1% and 1% solutions of trypsin (274 j./g, Wytwórnia Surowic i Szczepionek, Warszawa) prepared in a Sørensen's buffer (pH 7.6). The degree of female digestion was checked under a binocular every 5 hours. The suspension obtained after complete digestion and containing eggs was purified from proteases by washing several times with PS.

The eggs isolated by means of various methods were suspended in PS and incubated at 20°C. Each day, 3 drops were taken from each culture and the percentage of eggs in a specified developmental stage was determined under a microscope (Biolar, magnification 12.5×20). On the last day of incubation, the percentage of hatched larvae was counted based on the number of empty egg shells. The following stages were identified in the microscopic picture: cleavage (C), gastrulation (G), immobile larva (L₁), mobile larva (L₂), larva newly hatched from egg shells (L). Non-fertilized eggs were not considered in the calculations.

Results

The method of egg isolation consisting in the incubation of mature females in Eagle's medium with the addition of bovine serum and pepsin appeared to be ineffective. After 3 days of incubation, no eggs were found. Likewise, no eggs were obtained through the incubation of the females in pepsin solutions. No digestion of the nematodes was observed after 3 days of incubation.

The results showing a development rate of eggs obtained by the pressing method (I), homogenization (II) and digestion with solutions of trypsin (III and IV), and those referring to the number of hatched larvae of *C. rudolphii* are presented in Table 1.

In the cultures in which the eggs were isolated, either with the method of pressing (culture I) or homogenization (culture II), on the first day of culture the percentage of eggs at the stage of cleavage accounted for ca. 2%. In both cultures, the rate of em-

Table 1. The development of *C. rudolphii* eggs obtained with the pressing method (I), homogenization (II), digestion with 0.1% (III) and 1% solutions of trypsin (IV)

Day of incubation	The stage of development	Method			
		I	II	III	IV
1	C	2 ± 2.0*	2 ± 2.6	15 ± 3.5	10 ± 2.5
2	C	5 ± 1.5	8 ± 1.5	24 ± 5.0	15 ± 2.3
3	C	31 ± 4.6	26 ± 1.5	43 ± 3.5	37 ± 6.8
	G			12 ± 3.5	15 ± 3.6
4	C	57 ± 4.7	43 ± 5.7	15 ± 3.8	16 ± 3.5
	G	12 ± 2.0	4 ± 1.7	42 ± 2.3	33 ± 4.7
	L ₁			18 ± 5.9	13 ± 4.6
5	C	49 ± 5.9	36 ± 6.7	8 ± 4.0	12 ± 3.5
	G	29 ± 4.5	17 ± 4.2	53 ± 4.5	37 ± 3.8
	L ₁			26 ± 7.0	28 ± 5.5
6	C	15 ± 2.1	18 ± 2.6		10 ± 2.5
	G	44 ± 5.1	35 ± 3.8	2 ± 2.5	7 ± 2.0
	L ₁	15 ± 2.0	3 ± 0.6	64 ± 7.6	41 ± 7.2
	L ₂			22 ± 4.0	13 ± 3.6
7	C	3 ± 1.5	7 ± 4.4		7 ± 4.7
	G	18 ± 3.2	13 ± 6.1		10 ± 4.0
	L ₁	57 ± 3.8	37 ± 3.2	5 ± 5.0	4 ± 3.8
	L ₂			82 ± 6.1	57 ± 3.2
8	L ₁	5 ± 3.0	3 ± 3.1		
	L ₂	77 ± 2.3	47 ± 2.9	28 ± 5.5	15 ± 2.1
	L	7 ± 2.0	4 ± 1.7	52 ± 3.8	30 ± 4.2
9	L	53 ± 4.0	38 ± 1.5	53 ± 3.5	31 ± 3.2

Explanations: C—cleavage; G—gastrula; L₁—first stage larva; L₂—second stage larva; L—larvae hatched from the egg shell

* Mean % and standard deviation

bryo development was similar. In the culture in which the eggs were obtained with the method of pressing, a substantially higher percentage of eggs achieved successive developmental stages compared to culture II – the microscopic picture showed that ca. 50% of eggs were damaged, demonstrating tangible changes in either shape or size. Eggs whose development stopped at the stage of cleavage or gastrulation were observed relatively frequently vacuolized in subsequent days. In turn, the undamaged eggs were developing appropriately and achieved successive stages in a similar time span as the eggs isolated by means of pressing. On day 9 of incubation, in culture II, the larvae left 38% of eggs shells. On the same day, in culture I, 53% of larvae left their egg shells, while vacuolisation of embryos at various developmental stages was observed in the remaining eggs.

The best method of isolating eggs of *C. rudolphii* turned out to be the digestion of nematodes with a 0.1% trypsin solution (culture III). Complete digestion of the nematodes was reached after three days of incubation. On the first day of the culture, 15% of the eggs were at the stage of cleavage. Those eggs were observed to reach the successive developmental stages considerably earlier than the eggs isolated with the other methods. On day 7 of incubation, 82% of the eggs were found to contain a mobile larva and on day 8 over 50% of the larvae had left the egg shells.

Using a 1% of trypsin solution, complete digestion of the females was achieved after 24 hours. On the first day of the culture, 10% of the eggs were at the stage of cleavage. In the subsequent days of culture, it was observed that in a number of eggs the process of development was stopped at the stage of

cleavage or gastrulation. On day 7 of the culture, 57% of the eggs were found to contain a mobile larva, and in the subsequent days ca. 31% of the larvae hatched from the egg shells. The embryos whose development had been arrested vacuolized.

Discussion

In this study, no eggs were isolated with the method of incubating mature females in a culture medium with the addition of pepsin – which has a stimulating effect on the maturation and egg laying by females [6]. It is the most physiological method that was exploited to isolate eggs of various nematodes [4–8]. Jeska and Caruso [4] described of conditions that should be assured in order to obtain fertilized eggs from females of *A. suum*. According to these authors, nematodes should be transported to a laboratory within 3 hours following the collection in a buffered physiological saline (PBS) with the addition of intestinal digesta. Incubation should be conducted at 37°C using a PBS pH 7.3 with the addition of 0.0015N sodium hydroxide, 11mM of glucose and 125 mg/l of gentamycin-sulfate as a culture medium. In an experiment by Kjøie [5], mature females of *H. aduncum* obtained from viviparous eelpots (*Zoarces viviparus*) were incubated in Petri dishes at a temperature of 5°C, in sterile salt water (10%). In turn, Iglesias et al. [6,7] and Adroher et al. [8] incubated mature females of *H. aduncum* and *A. simplex* at an atmosphere of 5% CO₂ using various culture media (RPMI, GLIT) additionally supplemented with serum and pepsin. The reason for the failure of that method in this study might have been an excessively long period from the shooting of the cormorants to collecting females and fixing them in warm physiological saline.

The method based on the homogenization of whole mature females used in this study, caused damage to egg shells. The first-ever application of this method to isolate the so-called “thin-shell” eggs of *A. simplex* was thoroughly described by Højgaard [11]. Homogenization with an A 518–519 electronic blender by Kenwood (at maximum speed for 4 minutes) was applied to whole nematodes with a mean length of ca. 4 cm. A homogenate containing eggs was purified by 3-fold rinsing in sea water, followed by centrifugation and filtration. Pure eggs obtained in this way were then incubated in sea water. According to this author, the method does not damage eggs and has no negative effect on embryogenesis. In the reported study, the eggs were isolated following the procedure described by the above-mentio-

ned author, nevertheless, a higher number of damaged eggs was observed, with a changed shape that did not undertake development. The stage of larva was reached by ca. 40% of the eggs, which is a good result taking into account the vast number of eggs produced by the female.

The described method has been applied considerably more often to isolate eggs of the so-called geohelminths, whose egg shells have 4 layers: a mucopolysaccharide layer produced by the uterus and three layers produced by the egg cell, i.e.: vitelline, chitin and a lipid layer. These shells make an egg very resistant to a variety of environmental factors, including mechanical ones. For the homogenization of *A. suum*, Costello [9] recommends using a hand-operated homogenizer which, does not cause damage to eggs. In turn, Cleveland and Laurence [10] isolated eggs from uteruses of *A. suum* with the use of a low-revolution mechanical homogenizer. Eggs of *C. rudolphii*, like those of other nematodes belonging to the Anisakidae, are so-called “thin-shell” eggs and their shells contain an outer protein layer and an inner vitelline layer [16]. A lack of a chitin layer makes the eggs less resistant to such factors as freezing, long-term storage, drying out or to chemical or mechanical factors [16,19].

The presented study demonstrated that out of the methods applied, digestion of mature females with a 0.1% trypsin solution appeared the best method for the isolation of *C. rudolphii* eggs. The rate of the development of these eggs was rapid, and the stage of mobile larva was reached by ca. 82% of the eggs. A similar number of larvae was achieved from the eggs isolated with the method of pressing from uteruses of mature females. However, as compared to the method of digestion, this method is far more laborious. In the case of small nematodes, e.g. *C. rudolphii* (mean female body length – ca. 3 cm), the preparation of a uterus and the pressing of eggs requires high precision and takes much time, whereas tissue residues remaining in the culture enhance the development of protozoa and fungi. The application of a 1% solution of trypsin shortened that time to a considerable extent, but had a negative effect on the development of embryos. The arrestment of embryogenesis occurred at various stages, and in subsequent days the embryos vacuolized and degraded. The stage of mobile larva was reached by 57% of the eggs, whereas larvae were hatched from as little as 31% of the eggs and others lost mobility and vacuolized after a few days. This process was likely to be due to the damaging action of trypsin penetrating the egg shells.

The results obtained may be compared with the findings of Ocheńska [20]. While incubating eggs of *A. suum* in solutions of animal proteases (chymotrypsin, trypsin and maxatase P), this author did not observe their negative effect on the development of embryos. In turn, the arrestment of embryogenesis occurred in solutions of plant proteases (bromelain, ficin, papain). According to this author, protease inhibitors contained in the eggs have a protective effect on the embryo. The excess of animal proteases, as well as plant proteinase, that may not be bound by an inhibitor contained in eggs are toxic to the embryos. The highest concentrations of trypsin and chymotrypsin inhibitor were found in undeveloped eggs of *A. suum*, and in eggs freshly pressed out of uteruses, whereas smaller ones were in eggs at the stage of cleavage and gastrulation [21]. A rapid decrease in the concentration of the inhibitor occurred at the II larval stage when the "pulmonary" larvae were preparing for colonizing the gastrointestinal tract. No pepsin inhibitor was found in any stages of the embryogenesis.

One of the protective mechanisms of parasites of the gastrointestinal tract against digestion is the production of enzyme inhibitors. The presence of inhibitors of trypsin, chymotrypsin, pepsin and elastase was stated in bodies of *A. suum*, *A. galli* and *A. simplex* [22–24]. The available literature lacks reports on the occurrence of protease inhibitors in *C. rudolphii*. This nematode inhabits the stomach of the final host, thus it probably produces a pepsin inhibitor to avoid digestion. This may be confirmed by the digestion of females incubated in solutions of this enzyme. Likewise, resistance to the action of pepsin and the inhibiting effect on its activity were observed in the case of *A. simplex* larvae, which under natural conditions inhabits the stomach of marine mammals [25,26]. The complete digestion of females incubated in trypsin solutions is likely to indicate a lack of trypsin inhibitor.

In the case of parasitic nematodes, the eggs are the most resistant to the action of various factors, including digestive enzymes, hence the method of digestion used for the first time in this study may be applied to obtain eggs of parasites.

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