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Comparative analysis of different methods of staining the larvae *Haemonchus contortus*, *Mullerius* sp. (Nematoda, Strongylida) and *Strongyloides papillosus* (Nematoda, Rhabditida)

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Abstract

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We conducted an analysis of 14 methods of staining nematode larvae of the species *Haemonchus contortus* (Rudolphi**,** 1803), *Strongyloides papillosus* (Wedl, 1856) and *Mullerius* sp. It was established that alizarin red, brilliant blue, gentian violet and bromophenol blue did not colour the nematode larvae acceptably without heating. The most contrasting staining of the cuticle of *H. contortus*, *S. papillosus* and *Mullerius* sp. was achieved using brilliant blue, methylene blue, Ziehl's solution (with heating of preparations) and Lugol's solution (without change in temperature). The staining of the nuclei of the intestinal cells of *H. contortus* was the best by using Lugol's solution, while to the best reveal the morphological peculiarities of the gullet of *S. papillosus* and *H. contortus*, stains brilliant blue, methylene blue, brilliant green (with heating of preparations) and Lugol's solution (without change in temperature) have been observed. Differentiation of the nematodes of the gullet *H. contortus* and *S. papillosus* is possible through determination of the presence of the cuticle using brilliant green (with heating of preparations). The methods of differential diagnostics presented here allow near-patient testing of the larvae of the above-mentioned nematode species to be conducted without paralyzing them by formalin or spirit, which saves time in their identification.

Key words

identification of larvae, intestinal pulmonary parasites of ungulates, near-patient diagnostics of parasites

Introduction

Strongylida of wild and domestic animals are common in many countries of the world, including Ukraine (Boyko and Brygadyrenko, 2016). This has great ecological significance for these parasites affect the numbers of ungulates. Therefore methods of differentiation and identification are very valuable for monitoring changes in their population density and distribution in the wild (Kuzmina, 2012; Boyko, 2015; Boyko et al., 2009; Boyko et al., 2016). Some scientists have conducted epidemiological studies trichostrongylidae infections in young cattle (Tharaldsen and Helle, 1984), other studied physiopathologie of digestion in sheep with trichostongylidoses (Dakkak, 1984), *Dictyocaulus viviparus* infection in red deer (Corrigall, 1985), fenbendazole treatment for the prophylaxis of nematodiasis (the lungworm, *Dictyocaulus viviparus*, and trichostrongylidae) in grazing calves (Downey and Cawdery, 1992). However, a wide range of questions

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has remained under researched. Particularly, the registration of the intensity of staining of juveniles stages by different dyes can simplify the diagnostic. The identification is made according to peculiarities of morphology. Especial attention is paid to the body length, caudal end of the larvae's indusium, structure of the pharynx, the number and the shape of the intestinal cells, the shape of caudal end of the larvae. *H. contortus* larvae are 730 µm long, have 16 intestinal cells of triangular shape, which are positioned in two lines, the pharynx is a thin tube. This differentiates them from larvae of Strongylida of the airways. They are smaller, intestinal cells are imperceptible, and the caudal end has a thorn (Van Wyk et al., 2004; Van Wyk and Mayhew, 2013). Often dyes are added to the preparations (Andrews and Daniela, 1974; Jones and Khalil, 1984; Ogawa and Egusa, 1986; Ashton and Wirasinha, 1973; Srisuphanunt et al., 2009; Hansen and Perry, 1994; Baker, 2008; Ogawa and Egusa, 1986). The methods of staining by cotton blue or acid fuchsin lactophenol are used for identifying nematodes at all stages of development in plant tissue and at the same time for their exact localization. Staining with bromophenol blue allows all endoparasitic and semi-endoparasitic root-knot nematodes to be identified at all stages of development in roots. Stained green and dark-blue, the larvae and eggs can be clearly seen on unstained plant tissue (Franklin and Goodey, 1949). A mixture of picric acid and aniline blue can be used for staining sections of ligneous roots. In reddish-yellow plant tissue nematodes and eggs that have obtained a blue colour are clearly seen (Gundy et al., 1959; Mamiya and Kiyohara, 1972).

Currently, only obsolete methods of differentiation for larvae of Strongylida in mammals can be found in the literature. This involves their culturing and defining of morphological peculiarities such as body length, caudal end of indusium, number and shape of intestinal cells. Therefore methods of differential staining of Strongylida larvae need to be developed. The objective of our research is to use different stains to colour the larvae of different nematodes (*Haemonchus contortus* (Rudolphi, 1803), *Strongyloides papillosus* (Wedl, 1856) and *Mullerius* sp.) and thus facilitate their differentiation and identification.

Materials and methods

Animals and experimental conditions

In the experiment we used larvae of the following nematode species *Haemonchus contortus* (Rudolphi, 1803), *Strongyloides papillosus* (Wedl, 1856) and *Mullerius* sp. The larvae of *H. contortus* (third stage) and *S. papillosus* (different stages) were cultivated during 8 days at a temperature of 25 °C, while those of the *Mullerius* sp. (first stage) were collected from the excrement of small cattle.

Test chemicals

For their coloration we used 14 stains which are often used for identifying protozoa and nematode larvae (Enaida et al., 2006; Gregory et al., 2004; Houas et al., 2001; Lachheb et al., 2002; Mane, Babu, 2011; Myung Park et al., 2007; Namasivayam et al., 1998; Qamar et al., 2005; Selvakumar et al., 2004): alizarin red, brilliant blue, Turk's solution, brilliant green, methylene blue, amido black 10B, eozin, Ziehl's solution, Lugol's solution, gentian violet, Romanowsky-type, Sudan, bromophenol blue, orange G. We added 0.1 ml of solution containing live larvae (5–12 individuals) to 0.1 ml of stain solution. The larvae were distinguished from the excrement using Baermann Test (Zajac et al., 2011). We used 2 variants of the experiment:

- 0.1 ml of staining solution, 0.1 ml of preparation with live nematode larvae, temperature of 25 °C
- 0.2 ml of staining solution, 0.1 ml of preparation with live nematode larvae, temperature of 60 °C.

The following methods of preparing the stains were used:

1. 1% alizarin red (0.1 g alizarin red to 10 ml of distilled water)
2. 1% brilliant blue (0.1 g brilliant blue to 10 ml of distilled water)
3. Turk's solution (methylene blue to 3% of acetic acid)
4. 1% brilliant green (0.1 g brilliant green to 10 ml of 60% ethyl alcohol)
5. 1% methylene blue (0.1 g methylene blue to 10 ml of 60% ethyl alcohol)
6. 1% amido black 10B (0.1 g amido black 10B to 10 ml of distilled water)
7. 1% eozin (0.1 g eozin to 10 ml of distilled water)
8. Ziehl's solution (10 ml of saturated spirit solution of fuchsine, 90 ml of 5% carbolic acid)
9. Lugol's solution (5 parts of iodine, 10 parts of potassium iodide and 85 parts of water)
10. 1% gentian violet (0.1 g gentian violet to 10 ml of distilled water)
11. Romanowsky-type (0.1% azure solution and 0.1% of eosin solution)
12. 1% Sudan (0.1 g Sudan to 10 ml of distilled water)
13. 1% bromophenol blue (0.1 g bromophenol blue to 10 ml of distilled water)
14. 1% orange G (0.1 g orange G to 10 ml of distilled water).

Results

Staining of larvae without temperature fixation

The results of experiments without temperature fixation showed different degrees of staining of the larvae of *H. contortus*, *S. papillosus* and *Mullerius* sp. by the

different stains (Table 1). All studied species of nematodes were resistant to the stains alizarin red, brilliant blue, Turk's solution, amido black 10B, eozin, gentian violet, Romanowsky-type, Sudan, bromophenol blue and orange G. Their cuticle was penetrated by Lugol's solution. *H. contortus* larvae were also stained with brilliant green, methylene blue, Ziehl's solution. For *H. contortus*, a high degree of penetration was shown by brilliant green, Ziehl's solution and Lugol's solution. When brilliant green stain was used, the larvae *H. contortus* obtained a uniform green color. This stain penetrated not only below the cuticle, but also the intestine, the cells of which were stained the most intensively (Fig. 1a).

In contrast, after staining of *H. contortus* larvae with Ziehl's solution, the larvae obtained a pink colour, and the intestinal cells were poorly visible (Fig. 1b). When Lugol's solution was used, the larvae of *H. contortus* were coloured light-brown. Their cuticle and the marked cells of the intestine did not differ in the extent of staining (Fig. 1c).

Table 1. Results of staining the nematode larvae

Stain	Without temperature fixation			With temperature fixation		
	H. contortus	*S. papillosus*	*Mullerius* sp.	*H. contortus*	*S. papillosus*	*Mullerius* sp.
Alizarin red	–	–	–	++	++	++
Brilliant blue	–	–	–	++	+	+++
Turk's solution	–	–	–	–	–	–
Brilliant green	++	–	–	+++	+++	+
Methylene blue	+	–	–	+++	+++	+++
Amido black 10B	–	–	–	–	–	–
Eozin	–	–	–	–	–	–
Ziehl's solution	++	–	–	+++	+++	+++
Lugol's solution	++	+++	+	+++	+++	+++
Gentian violet	–	–	–	+	+	+
Romanowsky-type	–	–	–	–	–	–
Sudan	–	–	–	–	–	–
Bromophenol blue	–	–	–	++	+++	++
Orange G	–	–	–	–	–	–

Extent of staining: +++, intense; ++, average; +, insignificant; – –, not stained.

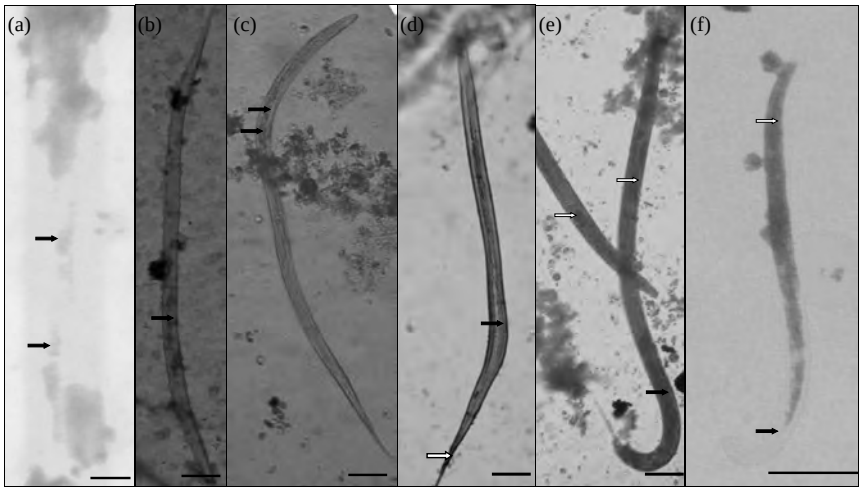

Fig. 1. Results of staining nematode larvae with brilliant green, Ziehl's solution, Lugol's solution, methylene blue, without temperature fixation: (a) – *Haemonchus contortus* (Rundolphi, 1803) stained with brilliant green (the pointer indicates cells of the intestine), (b) – *H. contortus* stained with Ziehl's solution (the pointer indicates cells of the intestine), (c) – *H. contortus* stained with Lugol's solution (the pointer indicates cells of the intestine), (d) – *H. contortus* stained with methylene blue (the black pointer indicates the intestine, the white one points to the caudal end of the cuticle), (e) – *Strongyloides papillosus* (Wedl, 1856) stained with Lugol's solution (the black pointer indicates the intestine, the white one points to the pharynx), (f) – *Mullerius* sp. stained with Lugol's solution (the black pointer indicates the caudal end, the white one points to the apical) end); bar = 10 µm (a) or 50 µm (b–f).

Methylene blue gave the larvae a paler colour. Unlike the results of the previous experiments, this stain penetrated only the cuticle and produced a violet tinge. The caudal end of *H. contortus* became dark violet. The intestinal cells of the larvae remained uncoloured.

When brilliant green was used, the larvae of *H. contortus* were viable, but became less active. Excellent results were given by methylene blue, Ziehl's solution and Lugol's solution: the larvae remained motionless, the cells of their intestines were visible.

The integuments of *S. papillosus* and *Mullerius* sp. let through only Lugol's solution. The larvae of *S. papillosus* (different stages) obtained a light-brown colour, their pharynx and the cells of their intestines were darker than the rest of their body (Fig. 1e). *Mullerius* sp. larvae were stained light-brown, their caudal ends were lighter than the intestines and apical ends (Fig. 1f).

Staining of larvae with temperature fixation

For staining of larvae with temperature fixation we used the same nematode species (Table 1). The colour of larvae intestine of all studied species did not change through Turk's solution, amido black 10B, eozin, Romanowsky-type and orange G.

A high extent of coloration among all studied species was shown by methylene blue, Ziehl's solution and Lugol's solution. When methylene blue was used, *S. papillosus* larvae obtained a very dark colour in their pharynx and intestine, which differed them from larvae of *Mullerius* sp. and *H. contortus*. Among larvae of *Mullerius* sp. the whole body was observed to be of a uniform bright blue colour (except the caudal end, which had a light colour, Fig. 2a, b). The larvae of *H. contortus* were also coloured to blue when Ziehl's solution and Lugol's solution were used and the preparations heated, all larvae turned bright pink (Fig. 2c) and brown.

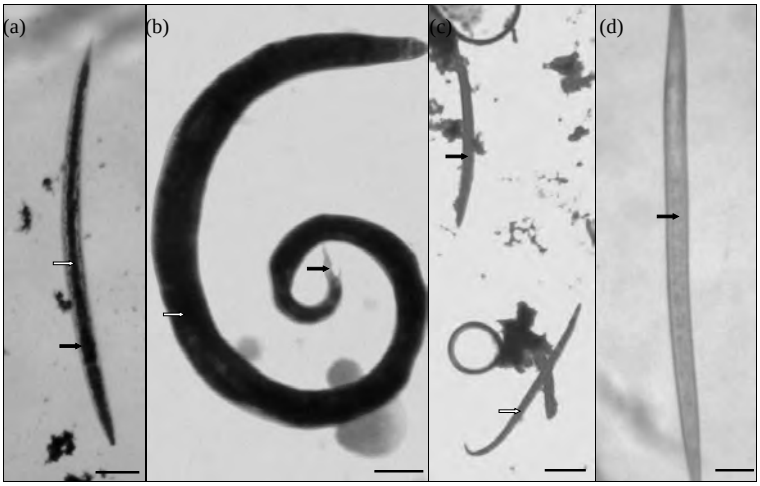

Fig. 2. Results of staining nematode larvae with methylene blue, Ziehl's solution, alizarin red with temperature fixation: (a) – *S. papillosus* stained with methylene blue (the black pointer indicates the pharynx, the white one point to the intestine), (b) – *Mullerius* sp. stained with methylene blue (the black pointer indicates the caudal end, the white one points to the intestine), (c) – *S. papillosus* and *Mullerius* sp. stained with Ziehl's solution (the black pointer indicates *S. papillosus*, the white one points to *Mullerius* sp.), (d) – *H. contortus* stained with alizarin red (the black pointer indicates the intestinal cells); bar = 10 µm (b) or 50 µm (a, c, d).

The stain alizarin red showed different results: the larvae of studied species were also coloured identically but with less intensity. They obtained a light-pink tinge (Fig. 2d). Other agents penetrated the cuticles of the larvae to different degrees. Larvae of *Mullerius* sp. were most strongly affected by staining with brilliant blue when heated, their bodies being uniformly coloured bright-blue. Larvae of *H. contortus* obtained a slightly brighter colour, but the cuticle, pharynx and intestinal cells were also uniformly coloured (Fig. 3a, b).

In the experiment with brilliant green, the brightest colours were obtained at *H. contortus* and *S. papillosus. S. papillosus* larvae obtained a strongly visible bright-green colour on the whole body and dark-green on the gullet and intestine. The integuments of *Mullerius* sp. larvae were characterized by a lesser penetrating capacity for this stain. Their larvae obtained a uniform light-green colour (Fig. 3c, d). The bodies of *H. contortus* larvae were uniformly coloured bright-green and the apical and caudal ends obtained a slightly brighter colour (Fig. 3e, f).

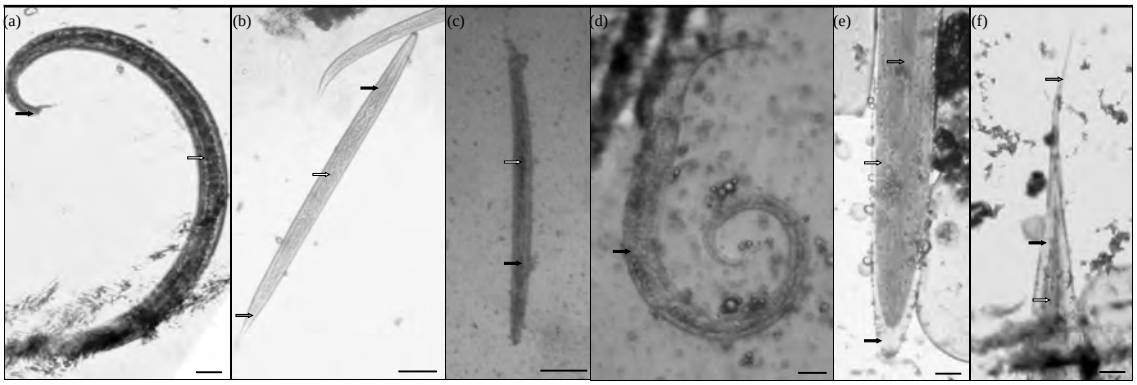

Fig. 3. Results of staining nematode larvae with brilliant blue, brilliant green with temperature fixation: (a) – *Mullerius* sp. stained with brilliant blue (the black pointer points to the caudal end, the white one indicates the intestine), (b) – *S. papillosus* stained with brilliant blue (black pointer indicates the pharynx, the white one points to the intestine, the grey one indicates the caudal end), (c) – *S. papillosus* stained with brilliant green (the black pointer indicates the pharynx, the white one points to the intestine), (d) – *Mullerius* sp. stained with brilliant green (the black pointer indicates the intestine), (e) – apical end *H. contortus* stained with brilliant green (the black pointer indicates the cuticle, the white one indicates the larva body, the grey one points to the pharynx), (f) – caudal end *H. contortus* stained with brilliant green (the black pointer points to the cuticle, the white one indicates the larva body, the grey one indicates the caudal end of the cuticle); bar = 10 µm (a, d–f) or 50 µm (b, c).

Similar results were received from staining larvae with bromophenol blue: the brightest colour was obtained by larvae of *H. contortus* and *S. papillosus*. The maximum extent of coloration was seen in *S. papillosus*. The cuticles of *Mullerius* sp. were also affected by this stain when the preparations were heated, though the extent of coloration was lower. Among the *Mullerius* sp. the colouring was not uniform: only the apical end of the larvae became bright-blue, the rest of the body was pale-blue (Fig. 4a–c).

In comparison with previously mentioned stains, the cuticles of the larvae of the three studied species of nematodes turned out to be resistant to gentian violet. Their bodies assumed a violet tinge. We observed that the larvae of *S. papillosus* changed colour only in the area of the pharynx and intestinal cells. The larvae of *Mullerius* sp. obtained a uniform violet tinge, and the cuticles of *H. contortus* larvae were coloured only in the area of the intestine, the caudal end did not absorb the agent (Fig. 4d–f).

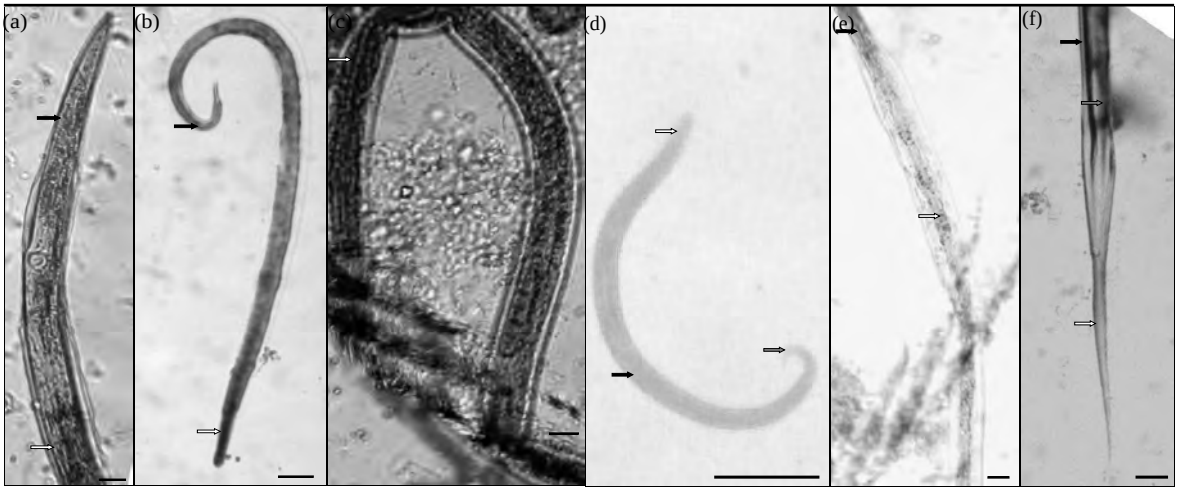

Fig. 4. Results of staining nematode larvae with bromophenol blue, gentian violet with temperature fixation: (a) – *H. contortus* stained with bromophenol blue (the black pointer indicates the apical end, the white one points to the intestine), (b) – *Mullerius* sp. stained with bromophenol blue (the black pointer indicates the caudal end, the white one indicates the apical end), (c) – *S. papillosus* stained with bromophenol blue (the white pointer indicates the apical end), (d) – *Mullerius* sp. stained with gentian violet (the black pointer indicates the intestine, the white one points to the apical end, the grey one points to the caudal end), (e) – *S. papillosus* stained with gentian violet (the black pointer indicates the pharynx, the white one points to the intestinal cells), (f) – *H. contortus* stained with gentian violet (the black pointer indicates the cuticle in the intestinal area, the white one points to the caudal end of the cuticle, the grey one points to the intestine) bar = 10 µm (a–c, e, f) or 50 µm (d).

The larvae of *S. papillosus* considerably differ in size at different stages of development. During the first hours their size corresponds to the size of nematode larvae in the windpipes of small cattle – *Mullerius* sp. For this reason, they are difficult to differentiate using microscopes of low magnification. When increasing the magnification, the number of microscopic fields also increases. Therefore identification and differentiation of larvae requires more time. For differentiation of these two species of nematode larvae Lugol's solution can be used. When using it, the preparation temperature should not be changed. Larvae of *S. papillosus* uniformly obtain a brown colour, and the larvae of *Mullerius* sp. obtain a light-brown (Table 2).

Table 2. Differential diagnostics of *S. papillosus* and *Mullerius* sp. larvae when using Lugol's solution

Characteristics	*S. papillosus*	*Mullerius* sp.
Colouring of the apical end	Light-brown	Brown
Colouring of the pharynx	Different from body colour, dark-brown	Not different from body colour, brown
Colouring of the intestine	Different from body colour, dark-brown	Not different from body colour, brown
Colouring of the caudal end	Uniform light brown	Different from body colour, light-brown

For differentiation of nematode larvae in the gullet, specifically representatives of Strongylida (*H. contortus*) and Rhabditida (*S. papillosus*), methylene blue, brilliant green, Ziehl's solution and Lugol's solution can be used. For identifying larvae it is enough to use only one of these agents. Staining using this method does not require heating (Table 3).

Table 3. Differential diagnostics of *H. contortus* and *S. papillosus* larvae using staining

Characteristics	*H. contortus*				*S. papillosus*			
	1	2	3	4	1	2	3	4
Colouring of the apical end	Blue	Green	Pink	Brown	Not coloured	Not coloured	Not coloured	Light-brown
Colouring of the pharynx	Blue	Green	Pink	Brown	Not coloured	Not coloured	Not coloured	Dark-brown
Colouring of the intestine	Not coloured	Dark-green	Pink	Cell membrane and nucleus dark-brown	Not coloured	Not coloured	Not coloured	Dark-brown
Colouring of the caudal end of the cuticle	Dark-blue	Green	Pink	Brown	Not coloured	Not coloured	Not coloured	Bright-brown
Colouring of the cuticle	Blue	Green	Pink	Brown	–	–	–	–

1, methylene blue; 2, brilliant green; 3, Ziehl's solution; 4, Lugol's solution.
Dash for *S. papillosus* means absence of cuticle.

Thus, larvae of *H. contortus* are coloured by first three agents shown in Table 3, while the larvae of *S. papillosus* remain uncoloured when treated by these agents. When Lugol's solution is used, larvae of *H. contortus* and *S. papillosus* do change their colour. The larval stages of *S. papillosus*, similar in size to those of *H. contortus*, are coloured bright-brown with a pronounced dark-brown hue to the pharynx and intestine. In contrast to *S. papillosus* the colour of *H. contortus* is brighter. For differentiation of *H. contortus* and *S. papillosus* brilliant blue can also be used, as *H. contortus* obtains a blue colour, and *S. papillosus* a violet colour.

Discussion

Lugol's solution is used for identification of nematode larvae (Dancescu and Mahjoub, 1981; Devaney et al., 1992; Amarante et al., 1999; McMurtry et al., 2000). Using this solution it was possible to identify nematode larvae of *Bunostomum* sp. and *Gaigeria* sp. at the third stages (the internal structure was clearly seen). In differential diagnostics these larvae clearly differed (they quickly took on a uniform obtaining brown colour across their entire length) from others, their apical end was from the start coloured less intensively than the other parts of

their body (Van Wyk and Mayhew, 2013). Lugol's solution was used for identifying *Cooperia oncophora* (Railliet, 1898), *Ostertagia ostertagi* (Stiles, 1892), *Strongyloides stercoralis* (Bavay, 1876) (DeRosa et al., 2005, 2008; Larsson et al., 2007; Areskog et al., 2014; Afzal and Steven, 2001).

One of the methods of lung strongyloidosis diagnostics (*S. stercoralis* filariform) is to stain the saliva sample with Gram. In a slide of human faeces stained with auramine O a larva of *S. stercoralis* was observed under ultra-violet light (Afzal and Steven, 2001).

Apart from differential diagnostics, the staining method was also used for defining the viability of nematodes using a solution of malachite green in the proportion 50 mg of preparation to 100 cm^3 of distilled water. Dead larvae were intensively coloured green with a blue tinge, and live larvae were much paler and were not coloured at all (Hastings and Bosher, 1938; Williams et al., 1989).

The identification of species of microfilaria is performed using coloured slides or thick drops of blood. The preparations are dried out, then hemolyzed and coloured according to Romanowsky-Giemsa, Wright (the cuticle of the microfilaria obtains a pale-violet colour, and the nucleus substance of the body a dark violet colour). The Bella method is most efficient for counting microfilaria in blood, where the Romanowsky-Giemsa or the hot haemotoxilin colour filter are used (Martínez-Palomo and Martínez-Báez, 1977; Kozek et al., 1983; Tocidlowski et al., 2000; Egyed et al., 2001).

Therefore, when using Turk's solution, amido black 10B, eozin, Romanowsky-type, Sudan and orange G nematode larvae of the digestive tract and in windpipes do not become coloured without heating the preparation. No data have been found concerning the usage of these stains for identification or differentiation of larvae and eggs of nematodes.

Alizarin red is also not a typical stain in parasitology. We discovered that nematode larvae are capable of being penetrated by the agent through their cuticle. One of the main conditions of such staining is fixation of the larvae by heating the preparation to 55 °C. After using alizarin red, larvae become pale-pink.

Brilliant green is used in parasitology for better viewing of the micropreparations with endameras. No research has been conducted on the usage of this stain for identifying Strongylida larvae. Our results have shown that *H. contortus* larvae can absorb this agent through their integuments. When the temperature is raised the larvae become bright-green with a light-green cuticle, and the apical and caudal ends of the cuticle are tinged light-green, while the larvae themselves become bright-green. Larvae of the intestinal nematode *S. papillosus* and the nematode of the air passages *Mullerius* sp. can be coloured when heated.

In the literature we found no data covering the usage of brilliant blue for staining parasitic objects. According to the results of our studies, Strongylida larvae (*H. contortus* and *Mullerius* sp.), when heated with brilliant blue, unlike the larval stages of Rhabditida, obtain a uniform bright-blue colour. Their pharynx and intestine obtain a light-violet tinge. Adding brilliant green to a preparation with *H. contortus* colours them light-green, for which with no heating is required. *S. papillosus* and *Mullerius* sp. obtain a green colour only when heated.

Similar results were shown by the experiment with methylene blue. When added to an unheated preparation, it coloured the larvae of *H. contortus*. Around the world this stain is used for staining parasites which are localized in the blood system. The data covering the staining of *Dictyocaulus* larvae by methylene blue indicate that it can be used to differentiate them from other lung nematodes. *Dictyocaulus* larvae obtained a violet colour, the rest remained uncoloured. According to the results of our experiment, larvae of other species of nematodes of the air passages can also be coloured, but only after heating. Then, larvae of *Mullerius* sp. obtain a bright-blue colour. In the literature, no data covering identification of *S. papillosus* larvae using methylene blue were found either.

H. contortus larvae become coloured even before heating. One of the stains around the world is Lugol's solution. It is capable of staining Strongylida helminth larvae. It is often used for improving the reliability of larvae identification. According to the results of our experiments, this stain gives the larvae of *H. contortus*, *Mullerius* sp. and *S. papillosus* a brown tinge (heating of the preparation is not necessary).

Ziehl's solution and gentian violet are not used for identifying parasites. In our experiment when the preparations are heated, Ziehl's solution, colours *H. contortus*, *Mullerius* sp. and *S. papillosus* pink. Adding gentian violet to the nematode larvae of the studied species does not produce results without heating. The larvae become coloured only with an increase in temperature (only the *Mullerius* sp. larvae become fully coloured, the *S. papillosus* larvae become coloured only in their digestive system).

Bromophenol blue is often used in phytoparasitology. This stain has not proved useful in identifying animal parasites. According to our data, when bromophenol blue is used, *H. contortus*, *Mullerius* sp. and *S. papillosus* larvae become coloured only after the preparation has been heated. *H. contortus* and *S. papillosus* obtain a uniform bright-blue colour. The larvae of *Mullerius* sp. were seen to become coloured only in the area of the apical end of the body. The rest of the body became pale-blue.

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