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A SIMPLE TECHNIQUE FOR STAINING OF PLATYHELMINTHS WITH THE LACTOPHNOL COTTON BLUE STAIN

By

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Abstract

This paper describes a simple technique for staining of flatworms using lactophenol cotton blue (LPCB). The staining was tested on 2 trematode species: *Heterophyes heterophyes* and *Mesostephanus appendiculatus*, and one cestode: *Diplopylidium acanthotetra*, which were collected from the intestine of stray cats in Kuwait. The specimens were mounted in a small amount of the LPCB stain on a clean slide for 2-3 minutes before covering with a cover slip. The technique rapidly and clearly differentiated the internal structures of the helminthes. Its speed and simplicity are advantages over other staining methods. It is easily used in wide-scale surveys where a large number of platyhelminths have to be identified and it is suitable for field studies.

Key words: LPCB stain, flatworms, cats, Kuwait

Introduction

Platyhelminths are found as adult or larval stages in various organs, particularly in the intestinal organs of many animal species including man (Pulis and Overstreet, 2013). Proper identification of these parasites is often vital for a correct diagnosis, morphological studies, systematic studies, zoogeographical studies and in additions, for the systematic collection of parasites (Cribb and Bray, 2010). The classical identification of flatworms is based on morphological features such as body size, scolexes, suckers, hooks, spines, testes, ovaries, cirrus sacs, vitelline glands etc. Therefore, it is needed that internal structures are stained visible and distinguishable for identification (Zainon *et al*, 2012).

Many stains and procedures have been used for trematode and cestode identification, each has its own technique, advantages and problems. Often, however, the procedures of staining require time, several steps, many chemicals and skills. In countrywide surveys, such the current survey of parasites of stray cats in Kuwait, large numbers of parasites are to be examined. This survey requires a quick identification and diagnosis, and consequently, a rapid and reliable staining technique (Carvallo *et al*, 2011).

This communication presents a simple technique for staining of platyhelminths with lacto-phenol cotton blue (LPCB).

Material and Methods

The staining was tested on two trematode species, *Heterophyes heterophyes* and *Mesostephanus appendiculatus*, and one cestode*, Diplopylidium acanthotetra*. Helminths were taken from the small intestine of stray cats that were part of a survey of parasites of stray cats around Kuwait.

After removal of the worms from the intestinal contents, the trematodes were washed in saline and fixed in 70 % alcohol. The trematodes were left as such. The strobila of the *Diplopylidium* worm was cut into small parts; each is composed of several segments, flattened between 2 slides and fixed in 70% alcohol till staining.

Procedures: 1- According to the size of the specimen place suitable drops of the LPCB stain ready-for-use (Merck, Germany) on a clean slide. 2- Immerse the trematodes or

the scolexes in the LPCB mountant / stain. 3- Strobila parts of the cestode were removed from the fixative and manually flattened in the stain on the slide. 4- After 3 minutes, cover the preparations with cover slips. 5- For permanent preparations seal the adages of the cover slip with nail polish.

Results

With this staining method, the anatomical structures of *H. Heterophyes*, M. *appendiculatus* and *D. acanthotetra* were clearly visible and well differentiated. They appear blue in color with different grades. Details are given in figures (1, 2, 3 & 4).

Discussion

In the present study, after 6 months follow-up, no changes were noted and the stain still kept its properties.

According to Pritchard and Kruse (1982) important considerations in the choice of a staining technique, are (i) its ability to differentiate clearly the anatomy of the specimens, (ii) its easy use and dependability, (iii) its compatibility with fixing agents, and (iv) its ability to retain optimum staining. The LPCB technique fulfilled these criteria as a good stain for platyhelminths.

It stains the internal structures of *M. appendiculatus*, *H. heterophyes*, and *D. acanthotetra* to be visible clearly for identification, and the staining has lasted good so far for 6 months, the technique is simple and rapid, and can be used under field conditions. The latter advantage is important when running large-scale surveys with large numbers of specimens that need identification.

The technique has advantages over the use of hematoxyline and carmine, common stains for identification of flukes and tapeworms. Since the former is used in an aqueous solution and the latter is prepared in alcohol, the specimens must be passed through a graded series of concentrations of alcohol to the level of stain (Schmidt, 1986). The LPCB has an advantage over the two stains because specimens stained and mounted in it need not be dehydrated; this reduces the steps of staining and thus saves time and gives rapid results. It also reduces chemicals and subsequently the costs of the technique.

The LPCB stain is a combined fixative, staining and clearing agent has four compounds: (i.) phenol, which kills living organisms and deactivates lytic cellular enzymes, (ii.) lactic acid which preserves (fungus) structures and (iii.) cotton blue, an acidic dye, which stains the chitin in fungal cell wall. Finally, (iv.) glycerol provides a semi-permanent preparation (Leck, 1999; Parija *et al*, 2003). As shown in this study, the tissues of flukes and tapeworms have a different affinity for LPCB, which is an acid stain. The acid removes some cytoplasm elements, making the parasites more receptive to stains (Pritchard and Kruse, 1982). The technique is widely used in wet mount preparations of fungal specimens (Thomas *et al*, 1991) and in the detection of the intestinal parasites in stool samples (Parija and Prabhakar, 1995; Parija, 1998; Parija *et al*, 2003). The stain has also been used in lengthy procedures and in combination with fuchsin-red for permanent mounts of flatworms (Dass, 1949).

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Explanation of figures

Fig. 1: Shows stained adult *M. appendiculatus*, testes are large, ovoid, tandem in position and situated in the posterior body half. Nearly pyramidal ovary is situated postero-lateral to the anterior testis. Gonads are stained light blue. Subterminal oral sucker and pharynx are well developed and deeply stained, whereas the feeble acetabulum lightly stained and lies in the middle of

the body. Vitellaria are well developed, deeply stained, irregularly shaped of closely and packed follicles, confined in horseshoe manner around the gonads. Cirrus sac is deeply stained, lies in the posterio-lateral body region, and contains the seminal vesicle and rod shaped cirrus.

Fig. 2: Shows stained whole worm of *H. heterophyes*. Oval-shaped testes are situated in the posterior part of the worm. Oval ovary is situated in the median field in front of the testes. The gonads appear light blue in color compared to the vitelline glands which are deeply stained blue occupying the posterior lateral field of the worm. Ventral sucker appears in the middle of the body, twice larger than oral sucker; both of them are deeply stained. Genital sucker is postro-lateral to the ventral sucker, deeply stained and provided with about 77 rod-let spines.

Fig. 3: Shows mature segment of *D. acanthotetra* with 2 sets of genital organs. Ovary is bilobed in shape and the two compact lobes are separated by the deeply stained seminal receptacle. The vitelline gland is situated posterior to the ovary. Vagina crosses the cirrus pouch and opens anterior to the male genital opening. Testes are numerous, rounded and situated in the middle field of the segment. The convoluted vasa defernses are deeply stained and situated in the upper part of the segment. Cirrus pouch is saccular containing the deeply stained cirrus.

Fig. 4: Shows *D. acanthotetra* scolex is provided with 4 suckers and rosttelum which had 4 alternating rows of deeply stained hooks, the first 2 rows of taenioid shaped hooks and the last 2 rows of small rose-thorn shaped hooks.

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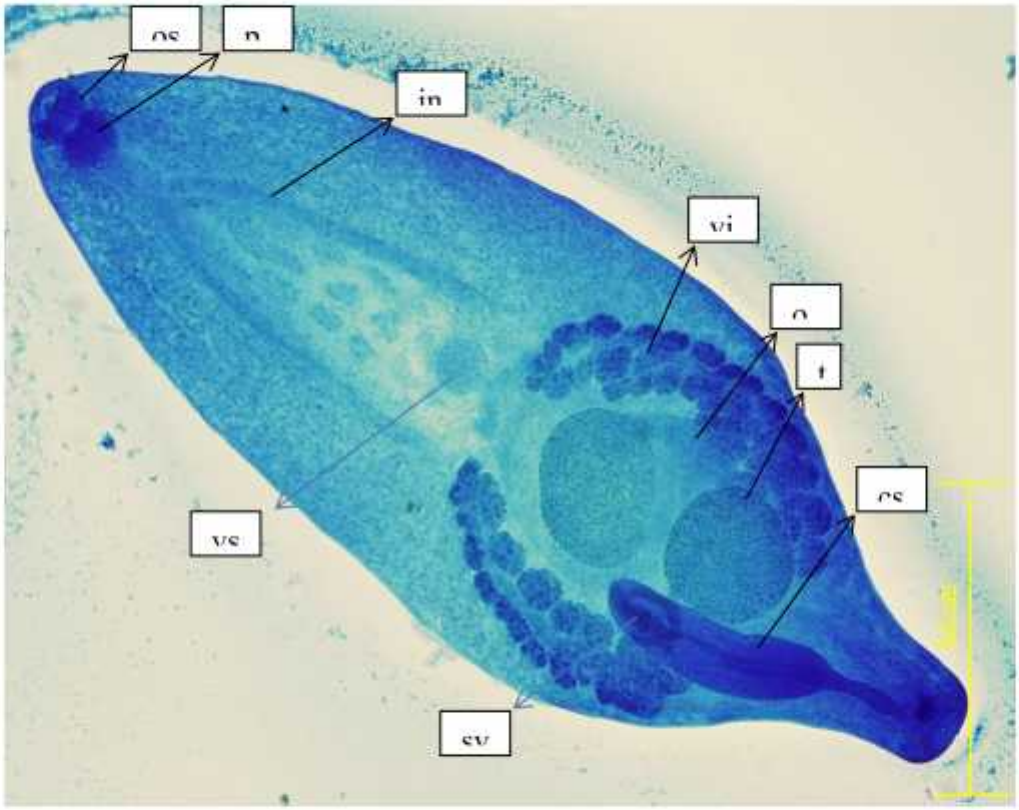

Fig. 1: *Mesostaphanus appendiculatus* (adult worm) x100 , os – oral sucker; ph –pharynx; int – intestinal caecum; vit – vitellaria; ov – ovary; t – testis; cs – cirrus sac; sv – seminal vesicle; vs – ventral sucker

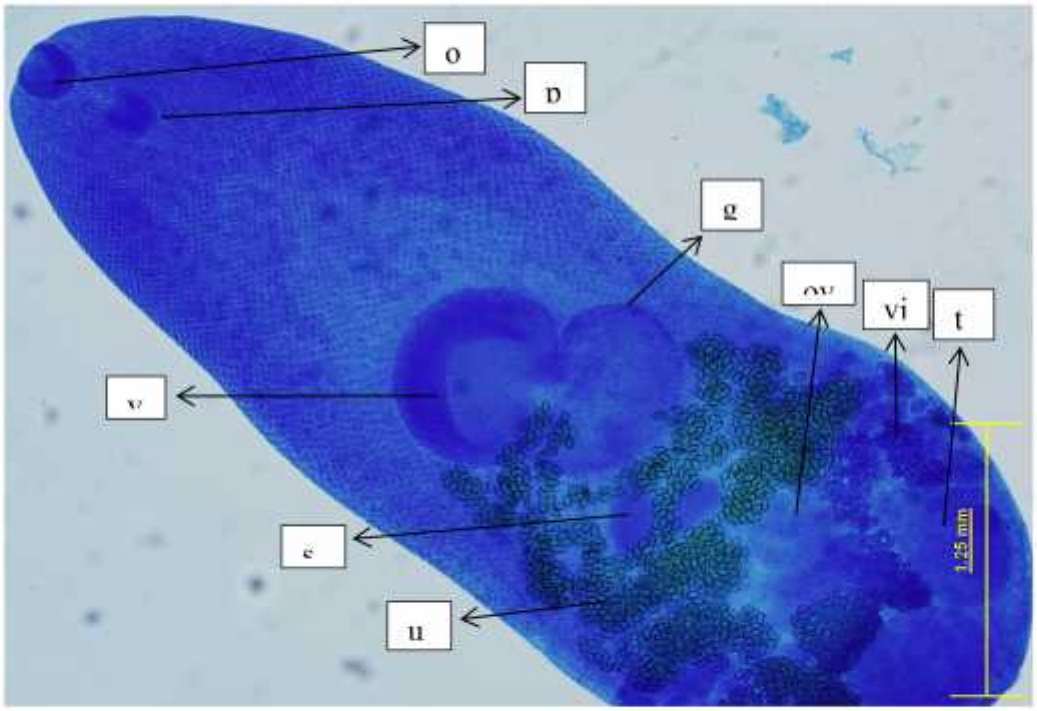

Fig. 2: *Heterophyes heterophyes* (adult worm) x100. os – oral sucker; ph –pharynx; gs – genital sucker; ov – ovary; vit – vitellaria; vs – ventral sucker; t – testis; sv – seminal vesicle; ut – uterus

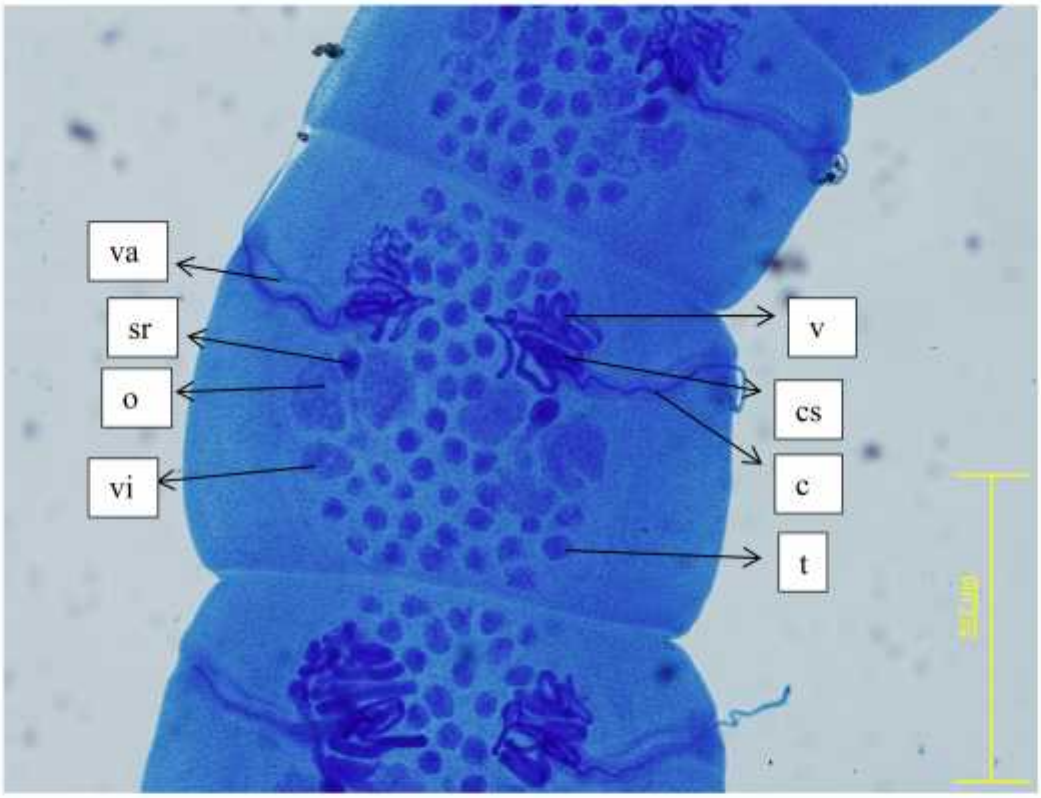

Fig. 3: *Diplopylidium acanthotetra* (mature seg.) x100 , vg- vagina; sr- seminal receptacle; ov- ovary; vit- vitellaria; vd- vas deferens; cs- cirrus sac; c- cirrus; t- testis

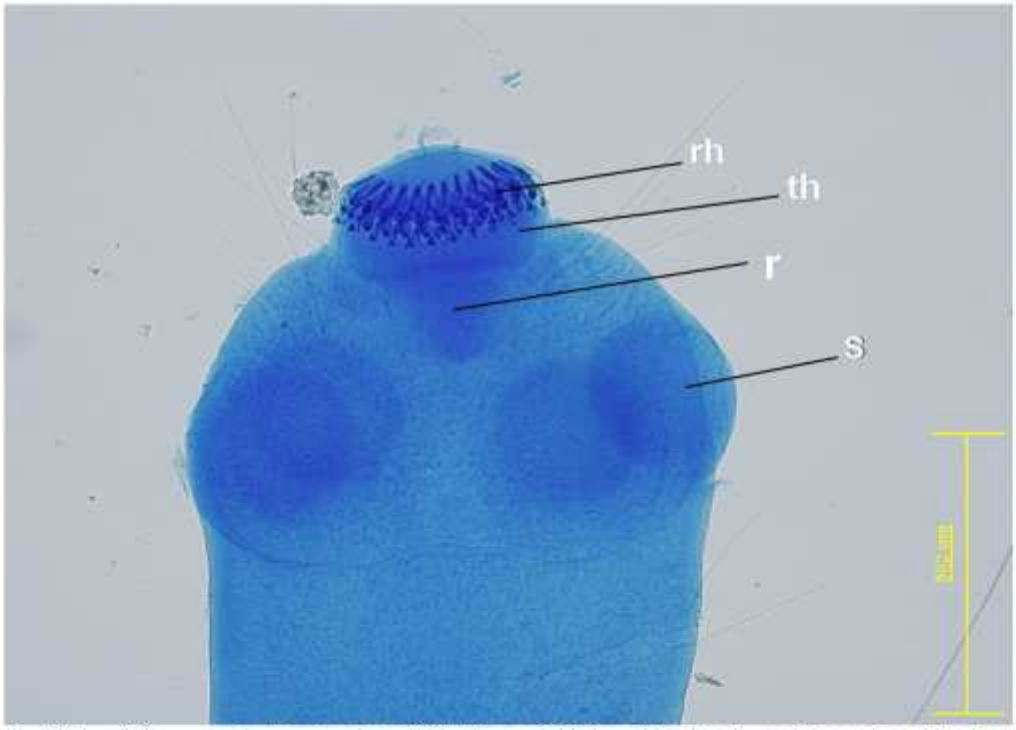

Fig.4: *Diplopylidium acanthotetra* (scolex) x200 th- taenioid shaped hooks; rh- rose thorn shaped hooks; r- rostellum; s- sucker;