Research Note

Comparative study among lactophenol blue, lactophenol solution and proteinase-K lytic solution for rostellar hooks morphometry of *Echinococcus granulosus* protoscolices

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Summary

Echinococcus granulosus is a tapeworm whose life cycle includes dogs and other canines as final hosts, while domestic and wild ungulates act as intermediate hosts for the tissue-invading larval stage (metacestode). *E. granulosus* has a worldwide geographical distribution. Protoscolices and rostellar hooks of *E. granulosus* are useful for diagnosis and rostellar hook morphometric features may be useful to discriminate *E. granulosus* and related species. The present study was aimed to determine a more suitable lytic solution and to obtain a clearest vision for performing morphometric studies on the rostellar hooks of *E. granulosus* protoscolices. Five fertile hydatid cyst samples were collected from sheep in Kirkuk slaughterhouse, Iraq, during June of 2015. According to the results of the present study, proteinase-K lytic solution is the best approach in morphometric analysis to get a clear vision of rostellar hooks and a safer usage in comparison with solutions containing lactophenol (lactophenol, lactophenol blue).

Keywords: *Echinococcus granulosus*; protoscolices; rostellar hook; morphometric study

Introduction

Echinococcus granulosus is a tapeworm whose life cycle includes dogs and other canines as final hosts for the intestinal tapeworm, while domestic and wild ungulates act as intermediate hosts for the tissue-invading larval stage, metacestode (Moro & Schantz, 2009). *E. granulosus* has a worldwide geographical distribution (Eckert & Deplazes, 2004). Hydatid disease (hydatidosis) is the larval infection characterized by long-term growth of hydatid cysts in the intermediate host. In internal organs, mainly liver and lungs of humans and other intermediate hosts, hydatid cysts of *E. granulosus* develop as unilocular fluid-filled bladders (McManus *et al.*, 2003). Hydatid cyst consists of external acellular laminated layer and internal nucleated germinal layer; the last one may give rise by

asexual budding to brood capsules. Protoscolices originate from the internal layer of the brood capsules (Thompson & McManus, 2001).

For morphometric study of rostellar hooks from *E. granulosus* protoscolices, a lytic solution must be used for digesting protoscolices and preparing the microscopic slides. Lactophenol blue solution is a common reagent used in mycology, but it is also very useful in evidencing protozoal cysts, trophozoites and ova of parasitic helminths, in addition to studying the internal structure of the parasitic pathogen (Jada *et al.*, 2016). Lactophenol blue solution contains cotton blue, which stains internal structures. Lactophenol blue and lactophenol solution contain phenol and lactic acid, which kill viable trophozoites, and may also kill protozoal cysts and helminthic eggs. Finally, glycerol in lactophenol blue and lactophenol solution

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provide semi-permanent preparations (Parija & Prabhakar, 1995). Proteinase-K is a main proteolytic enzyme, obtained upon purification from *Tritirachium album*. This enzyme was named "proteinase-K" with respect to its keratin hydrolyzing activity. Proteinase-K has a strong proteolytic activity- about six times more than pronase and about three times more than bovine trypsin- on denatured proteins, for example, hemoglobin and casein (Ebeling *et al.*, 1974). Protoscolices and rostellar hooks of *E. granulosus* are useful for diagnosis, also in relation to cysts vitality. In addition, rostellar hook morphometric features may be useful to discriminate the isolates of *E. granulosus* from the related species *E. canadensis* (Harandi *et al.*, 2012), and the morphometric features of rostellar hooks of protoscolices are useful in differentiation between strains of *E. granulosus* of different intermediate hosts (Mowlavi *et al.*, 2012; Arbabi *et al.*, 2017). The present study is aimed to compare different suitable lytic solutions in order to obtain a clearest vision for performing morphometric studies on the rostellar hooks of *E. granulosus* protoscolices.

Materials and Methods

Collection of samples

Out of 34 fertile hydatid cysts collected from 18 infected sheep in Kirkuk slaughterhouse, Iraq during June of 2015, five fertile hydatid cyst samples were selected from liver to perform the present study. According to Perez-Serrano *et al.* (1995), protoscolices were isolated from each hydatid cyst and washed with phosphate buffer saline (PBS), then stored in ethanol 70% tubes until used.

Preparation of slides for microscope

The study was designed for the first time for comparing among three lytic solutions (Lactophenol blue, lactophenol and proteinase-K

lytic) for preparation of microscopic slides. Lactophenol blue solution of Central Drug House company (India) was used. Lactophenol solution was prepared by mixing 50 ml of phenol (CDH, India), 10 ml of lactic acid (Solvo Chem, UK), 20 ml of glycerol (Solvo Chem, UK) with 900 ml of distilled water (Humason, 1979). Proteinase-K lytic solution was prepared according to Wu *et al.* (2007) with modification, by mixing 300 µl of TE9 buffer with pH 7.5 and 200 µl of 10 mg/ml of proteinase-K stock solution. TE9 buffer was prepared according to Wu *et al.* (2007) and Blasco-Costa *et al.* (2012) with modification on the volume of components. TE9 buffer contained 10 mM of tris-HCl (Scharlau, Spain), 125 mM of sodium chloride (SCRC, China) and 10 mM of EDTA (Panreac, Spain) with pH 8. Proteinase-K stock solution with 10 mg/ml (w/v)% was prepared according to Harvey (2000) by dissolving 50 mg of proteinase-K (Bio Basic, Canada) in 5 ml of distilled water (Alkem laboratories, India). Lactophenol blue, lactophenol and proteinase-K lytic solutions were used on all samples (for comparison) as in the following steps: i) one drop of protoscolices with ethanol 70 % was taken by 3 ml Pasteur pipette and laid on the middle of microscope slide for each solution and was examined under microscope before adding the lytic solutions to act time zero; ii) each slide was putted in separated Petri dish, and then a drop from each lytic solution was added on the specimens separately. All slides were incubated for 4 hours with repetitive microscopic examinations under 100x and 400x magnification every 30 minutes (time 30, time 60, time 90, time 120, time 150 and time 240 minutes) with continued repetitive additions of the all lytic solutions to prevent specimen drying. Slide with proteinase-K lytic solution was incubated at 56 °C, while, lactophenol blue and lactophenol solution slides were incubated at a room temperature; iii) at time 240, the incubation was stopped, and a drop of aqueous gelatin-glycerol was added to all specimens to preserve it for a long time. Aqueous gelatin-glycerol consisting

Table 1. Clustering behavior of protoscolices and visibility of components at different times after treated with lactophenol-based solutions and Proteinase-K lytic solution.

Time of exposure	Lactophenol blue or lactophenol solution	Proteinase-K lytic solution
Time 30 minutes	Protoscolices aggregated/ Components and rostellar hooks not visible (Fig. 2a-b)	Protoscolices separated/ clearer vision of components (Fig. 2c-d)
Time 60 minutes	Protoscolices aggregated/ Components and rostellar hooks not visible (Fig. 3a-b)	Protoscolices separated/ clearer vision of components (Fig. 3c-d)
Time 90 minutes	Protoscolices aggregated/ clearer vision of components (Fig. 4a-b)	Protoscolices separated/ vision of components same as time 60 (Fig. 4c-d)
Time 120 minutes	Protoscolices partially separated/ components visible but with no lysis of membrane (Fig. 5a-b)	Protoscolices well separated/ starting of membrane lysis (Fig. 5c-d)
Time 150 minutes	Protoscolices partially separated/ components visible but with no lysis of membrane (Fig. 6a-b)	Protoscolices well separated/ increasing of membrane lysis (Fig. 6c-d)
Time 240 minutes	Protoscolices partially separated/ components visible (better with lactophenol) but with no lysis of membrane (Fig. 7a-b)	Protoscolices well separated/ membrane lysis semi-completed (Fig. 7c-d). Rostellar hooks free and visible (Fig. 8)

Fig.1. Microscopic photography of protoscolices at different magnification (a, b) before use of lytic solutions (zero time), showing aggregation of protoscolices within the preservation solution (ethanol 70%).

of 5 g of gelatin (Panreac, Spain), 50 ml of glycerol (Solvo Chem, UK), 5 ml of phenol (CDH, India) and 50 ml of distilled water (Humason, 1979). In conclusion, a microscopic examination was carried out under 1000x with oil immersion and fine pressure on the coverslip was done to identifying the rostellar hooks.

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors therefor the present study formal consent is not required.

Results

During the first inspection, before adding the lytic solutions, the protoscolices resulted aggregated after examination using a microscope with 400x, thus not allowing a clear definition of the internal structures and rostellar hooks (Fig. 1a, b). Whereas, at the last step, after 240 min from the addition of lytic solutions, lactophenol blue provided still little clear vision and no lysis of protoscolices membranes even after putting a cover-slide on sample; lactophenol solution gave a more clear vision, the cellular components were noticed but also un-differentially and no lysis of cellular membrane even after putting a cover-slide on sample was observed; proteinase-K lytic solution determined the clearest vision with semi-complete lysis of cellular membrane and with complete lysis after putting a cover-slide on sample. After using proteinase-K lytic solution, the hooks appeared distributed in two separated rings alternatively between large and small hooks, and each one had three regions (blade, guard and handle). Also, after using proteinase-K lytic solution, it became easier to count the number of hooks (mean= 34, SD= 4) and to distinguish between large hooks (larger, less robust, had more pointed blade) and small hooks. Clustering behavior of protoscolices and visibility of internal components at each time after time zero are summarized in Table (1).

Discussion

In the present study, treatments of rostellar hooks of *E. granulosus* protoscolices with lactophenol blue and proteinase-K lytic solution were compared for the first time for morphometric analysis. Lactophenol solution or polyvinyl lactophenol have been used in several studies for morphometric characters on rostellar hooks of *E. granulosus* protoscolices, but no information about comparison with lytic solutions are so far available (Hobbs *et al.*, 1990; Constantine *et al.*, 1993; Almeida *et al.*, 2007; Karimi & Dianatpour, 2008; Almeida *et al.*, 2009; Yildiz & Gurcan, 2009; Calderini *et al.*, 2012; Harandi *et al.*, 2012; Soriano *et al.*, 2013; Fadakar *et al.*, 2015; Mustafa *et al.*, 2015).

According to Central Drug House (CDH), the manufacture company, lactophenol either in lactophenol blue or in lactophenol solution is harmful if swallowed or inhaled by humans, provoking severe skin burns and eye damage, and is suspected of causing genetic defects due to germ cell mutagenicity and may cause damages to organs following prolonged or repeated exposure.

Proteinase-K lytic solution is also frequently used in molecular studies for DNA extraction on various living cells and tissues. Also, proteinase-K lytic solution was used as enzymatic digestion technique to obtain and study the sclerotized structures of monogenean parasites as *Ligophorus* and *Solostamenides*, in addition to using it in molecular studies for the identification of these monogenean parasites (Hernández-Orts *et al.*, 2010; Blasco-Costa *et al.*, 2012; Rodríguez-González *et al.*, 2015, Al-Nasiri & Balbuena, 2018). So far, proteinase-K lytic solution was not used previously

Fig. 2. Microscopic photography of protoscolices after 30 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

Fig. 3. Microscopic photography of protoscolices after 60 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

Fig. 4. Microscopic photography of protoscolices after 90 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

Fig. 5. Microscopic photography of protoscolices after 120 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d); arrows indicated the sites of lysis in cellular membrane.

Fig. 6. Microscopic photography of protoscolices after 150 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d).

Fig. 7. Microscopic photography of protoscolices after 240 min from zero time of addition of lactophenol blue (a), lactophenol solution (b), proteinase-K lytic solution (c, d), arrows indicated the sites of lysis in cellular membrane.

Fig. 8. Microscopic photography of hooks after lysis the membrane of protoscolices after 240 min from zero time of addition of proteinase-K lytic solution to the protoscolices.

for morphometric study on rostellar hooks of *E. granulosus*. Additionally, in the present study, proteinase-K lytic solution made well available to the observer those morphometric features of high taxonomic values, such as large and small hook length and blade length, as described in previous papers (Kumaratilake & Thompson, 1984; Hobbs *et al.*, 1990).

The present study is the first study that evaluates lactophenol blue and proteinase-K lytic solution in digestion of *E. granulosus* protoscolices for morphometric study of rostellar hooks.

Based on the obtained results, the present study recommends the use of proteinase-K lytic solution in morphometric of rostellar hooks, as it provides a clearer vision of hooks if compared with lactophenol-based solutions and may guarantee safer conditions for operators, although a protective equipment must be used in any case.

Conflict of Interest

Authors state no conflict of interest.

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