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

S. J. HAMMAD¹, S. CAVALLERO², F. S. AL-NASIRI¹*, S. D’AMELIO²

¹Department of Biology, College of Science, Tikrit University, Tikrit, Iraq, E-mail: *fshnasiri@yahoo.com, salamalesamy@yahoo.com;
²Department of Public Health and Infectious Diseases, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy, E-mail: serena.cavallero@uniroma1.it, stefano.damelio@uniroma1.it

**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
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 different suitable lytic solutions in order to obtain a clearest vision for performing morphometric studies on the rostellar hooks of E. granulosus protoscolices.

| 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) |
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 (Hummason, 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.
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.

**References**

Almeida, F.B., Rodrigues-Silva, R., Neves, R.H., Gonçalves, M.M.L., Romani, E.L.S., Machado-Silva, J.R. (2009): Morphological and morphometric studies on protoscolices rostellar hooks of *Echinococcus granulosus* from Peru visualized by several microscopic techniques. *Neotrop. Helminthol.*, 3 (2): 65 – 72

Almeida, F.B., Rodrigues-Silva, R., Neves, R.H., Romani, E.L.S., Machado-Silva, J.R. (2007): Intraspecific variation of *Echinococcus granulosus* in livestock from Peru. *Vet. Parasitol.*, 143: 50 – 58. DOI: 10.1016/j.vetpar.2006.07.028

Al-Nasiri, F.S., Balbuena, J.A. (2018): Solostamenides iraqensis n. sp. (Monogenoidea, Microcotylidae ) parasitizing the freshwater mullet *Liza abu* (Pisces, Mugilidae) Iraq. *Vie et milieu*, 68 (4): 245 – 251

Arbabi, M., Pirestani, M., Delavari, M., Hooshyar, H., Abdoli, A., Sarvi, S. (2017): Molecular and morphological characterizations of *Echinococcus granulosus* from human and animal isolates on Kashan, Markazi Province, Iran. *Iran J. Parasitol.*, 12 (2): 177 – 187

Blasco-Costa, I., Miguez-Lozano, R., Sarabev, V., Balbuena, J.A. (2012): Molecular phylogeny of species of *Ligophorus* (Monogenea: Dactylogyridae) and their affinities within the Dactylogyridae. *Parasitol. Int.*, 61: 619 – 627. DOI:10.1016/j.parint.2012.06.004

Calderini, P., Gabrielli, S., Cancrini, G. (2012): Is the goat a new host for the G3 Indian buffalo strain of *Echinococcus granulosus*? *Sci. World J.*, 2012: 1 – 5. DOI: 10.1100/2012/286357

Constantine, C.C., Thompson, R.C.A., Jenkins, D.J., Hobbs, R.P., Lymbery, A.J. (1993): Morphological characterization of adult *Echinococcus granulosus* as a means of determining transmission patterns. *J. Parasitol.*, 79 (1): 57 – 61
Genotyping and phenotyping of Echinococcus, a zoonosis of increasing concern.

Clin. Microbiol. Rev., 17: 107 – 135. DOI:10.1128/CMR.17.1.107-135.2004

Fadakar, B., Tabatabaie, N., Boruj, H., Naghibi, A. (2015): Genotyping of Echinococcus granulosus from goats and sheep indicating G7 genotype in goats in the Northeast of Iran. Vet. Parasitol., 214 (1 – 2): 204 – 207. DOI:10.1016/j.vetpar.2015.09.029

Harandi, M.F., Hajialilo, E., Shokouhi, M. (2012): Larval hook length measurement for differentiating G1 and G6 genotypes of Echinococcus granulosus sensu lato. Turkiye Parazitol. Derg., 36: 215 – 218. DOI:10.5152/tpd.2012.52

Harvey, D. (2000): Nematode Analytical Chemistry. 1st Edition, McGraw-Hill Com. NY., USA, 798 pp.

Heranández-Orts, J.S., Ahuir-Baraja, A.E., Raga, J.A., Montero, F.E. (2010): A new species of Empiruhtotrema (Monogenean: Monocotylidae) from Pteromyelaus bovinus (Myliobatidae) from the Western Mediterranean. J. Parasitol., 96 (6): 1081 – 1085

Hobbs, R.P., Lymbery, A.J., Thomson, R.C.A. (1990): Rostellar hook morphology of Echinococcus granulosus (Batsch, 1786) from natural and experimental Australian hosts, and its implications for strain recognition. Parasitology, 101: 273 – 281

Humason, G.L. (1979): Animal Tissue Techniques. 4th Edition, W.H. Freeman and Company, San Francisco, 468 pp.

Jada, S., Raju, S., Jayakumar, K., Sahu, P. (2016): Incidence of intestinal parasitic infestation and anemia among school children in Ammapettai. Inter. J. Rec. Sci. Res., 7 (11): 14312 – 14316.

Karimi, A., Dianatpour, R. (2008): Genotyping and phenotyping characterization of Echinococcus granulosus of Iran. Biotechnology, 7 (4): 757 – 762

Kumaratilake, L.M., Thompson R.C.A. (1984): Morphological characterisation of Australian strains of Echinococcus granulosus. Int. J. Parasitol., 14 (4): 467 – 477

McManus, D.P., Zhang, W., Li, J., Bartley, P.B. (2003): Echinococcosis. Lancet, 362: 1295 – 1304

Mowlaei, G., Salehi, M., Eshraghian, M., Rokn, M.B., Fashi-Harandi, M., Mohajeran, E., Salahi-Moghadam, A. (2012): Morphometric differentiation between camel and sheep strains of Echinococcus granulosus using computer image analysis system (CIAS). Asia. Pac. J. Trop. Med., pp. 58 – 21

Mustafa, I., Shabaz, M., Asif, S., Khan, M.R., Saeed, U., Sadoq, F., Mehmood, T., Ahmed, H., Simsek, S. (2015): Availability, cyst characteristics and hook morphology of Echinococcus granulosus isolates from livestock (cattle, sheep and goats) in central Punjab, Pakistan. Kafkas Univ. Vet. Fak. Derg., 21 (6): 849 – 854. DOI:10.9775/kvfd.2015.13755

Parija, S.C., Prabhakar, P.K. (1995): Evaluation of lacto-phenol cotton blue for wet mount preparation of feces. J. Clin. Microbiol., 33 (4): 1019 – 1021

Perez-Serrano, J., Denegri, G., Casado, N., Bodega, G., Rodriguez-Casabeiro, F. (1995): Anti-tubulin immunohistochemistry study of Echinococcus granulosus protoscolices incubated with albendazole and albendazole sulphoxide in vitro. Parasitol. Res., 81: 438 – 440

Rodriguez-Gonzalez, A., Miguez-Lozano, R., Llopis-Belleguer, C., Balbuena, J.A. (2015): A new species of Ligophorus (Monogenea: Dactylogyridae) from the gills of the flathead mullet Mugil cephalus (Teleostei: Mugilidae) from Mexico. Acta Parasitol., 60 (4): 767 – 776

Soriano, S.V., Pierangeli, N.B., Pianciola, L.A., Mazzeo, M., Lazzerini, L.E., Debiaggi, M.F., Bergagna, H.F.J., Basualdo, J.A. (2013): The optimum cut-off value to differentiate Echinococcus granulosus sensu stricto from other species of E. granulosus sensu lato using larval rostellar hook morphometry. J. Helminthol., 89: 1 – 8. DOI: 10.1017/S0017316213000473

Thompson, R.C.A., McManus, D.P. (2001): Aetiology: Parasites and Life-Cycles. In: Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (Eds.). WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern. OIE, Paris, pp. 1 – 19

Yildiz, K., Gurcan, I.S. (2009): The detection of Echinococcus granulosus strains using larval rostellar hook morphometry. Turkiye Parazitol. Derg., 33 (2): 199 – 202