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MORPHOLOGICAL AND MORPHOMETRIC STUDIES ON PROTOSCOLECES ROSTELLAR HOOKS OF *ECHINOCOCCUS GRANULOSUS* FROM PERU VISUALIZED BY SEVERAL MICROSCOPIC TECHNIQUES

ESTUDIOS MORFOLÓGICOS Y MORFOMÉTRICOS EN GANCHOS ROSTELLARES DEL PROTOESCOLICES DE *ECHINOCOCCUS GRANULOSUS* DEL PERÚ VISUALIZADA POR VARIAS TÉNICAS MICROSCÓPICAS

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Abstract

This study was undertaken to expand the current knowledge of the morphology and morphometry of rostellar hooks of protoscoleces from the metacestode *E. granulosus*. Protoscoleces were isolated from livers and lungs of naturally infected ovines and bovines obtained from abattoirs in Peruvian provinces (Arequipa, Cuzco, Puno, Huancavelica and Junin). Bright-field microscopy, confocal laser scanning microscopy, differential interference contrast and variable pressure scanning electron microscopy were used. Morphometry was made using computer image analysis. The application of these assay indicated that the large hooks frequently had thin guards and an irregular surface between the guard and handle. Data also showed that the small hooks presented rounded and stout guards. The blade did not show any relevant feature. No clear morphological distinction was observed between large and small hooks. Phenotypical polymorphism was evident in the shape and size of hooks. In conclusion, the current data show that large and small rostellar hooks have morphological polymorphism. Because the application of this knowledge for taxonomic study is limited, for this end morphometry techniques are required. Our study demonstrated the usefulness of combining conventional and new morphological tools to help to solve unresolved matters with regards to rostellar hooks features.

Key words: *Echinococcus granulosus* – microscopycal techniques – morphology – morphometry - Peru- rostellar hooks.

Resumen

Esta investigación fue realizada para incrementar el conocimiento actual de los datos morfológicos y morfométricos de los ganchos rostelares de la forma larvaria (metacestode) de *Echinococcus granulosus*. Los protoescólices fueron aislados de hígados y pulmones de ovinos y vacunos infectados naturalmente obtenidos de los mataderos en los departamentos del Perú (Arequipa, Cuzco, Puno, Huancavelica y Junín). Se utilizó la microscopia de campo claro, la microscopia confocal, la microscopia electrónica de barrido y contraste de interferencia diferencial. La morfometría se realizó mediante el análisis de imagen computacional. La aplicación de estos ensayos indican que los ganchos grandes con frecuencia presentaban finos protectores y de superficie irregular entre el protector y el mango. Los datos también indicaron que los ganchos pequeños presentaron proctectores redondeados y robustos. La hoja no mostró ningún aspecto relevante. Se observó que no existe distinción morfológica clara entre los ganchos grandes y pequeños. Fue evidente diferencias fenotípicas en la forma y el tamaño de los ganchos. En conclusión, la comparación de la morfología de los ganchos rostelares grandes y pequeños mostró ciertas diferencias. Nuestro estudio demostró la utilidad de la combinación de herramientas tradicionales y nuevas para los estudios morfológicos y ayudar a resolver las cuestiones pendientes con respecto a la morfología de los ganchos rostelares.

Palabras claves: *Echinococcus granulosus* – Técnicas microscopicas – morfología – morfometría - Perú- ganchos rostellares.

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INTRODUCTION

Echinococcus granulosus is a common small tapeworm living in the small intestine of domesticated and wild carnivores (Ingold *et al*., 2001). The larval stage (metacestode) causes cystic echinococcosis (CE) that remains one the most health problem worldwide, especially in the sheep-rearing rural areas (Eckert & Deplazes, 2004; Moro *et al.*, 2004; Ahmadi & Dalimi, 2006). The metacestode are found in liver and lungs of sheep, cattle, camels, goats, pigs, buffalo and horses (Thompson & McManus, 2002).

The rostellar hooks are distributed in 2 rows, each have a blade, guard and handle region (Rogan & Richards, 1987). Scanning electron studies revealed that hooks of upper row (large hooks) are longer, more pointed, slightly curved and less robust compared with hooks of the lower row (small hooks). In addition, large hooks had a projecting part with a smooth surface, whereas small hooks had a serrated edge (Antoniou & Tselentis, 1993).

Based on the metacestode rostellar hooks morphology, phenotypic variations have largely been observed in *E. granulosus* from different species of intermediate hosts (Ponce-Gordo & Cuesta-Bandera, 1997; Tashani *et al.*, 2002; Turčeková *et al.*, 2003; Ahmadi, 2004; Almeida *et al.*, 2007). Evidences also showed that protoscoleces differed markedly in both the number and size of hooks, which are host-induced (Hobbs *et al.*, 1990).

There is often a need to collect images for recording metacestode rostellar hooks for both taxonomic and morphological studies in *E. granulosus*. Usually, unstained mounts of hydatid fluid sediment are examined. Diverse techniques as Ryan and modified Baxby stains were recommended for visualization under transmitted-light microscopy, whereas Ziehl-Neelsen stain under green excitation light was very useful for fluorescence microscopy (Clavel *et al.*, 1999). However, the potential value of new techniques does not seem to have been deeply realized for *E. granulosus* studies. Because no specific sample preparation protocols are required, variable pressure scanning electron microscopy now is widely used for morphological studies (Muscariello *et al.*, 2005). In this paper, new data on the morphology and morphometry of rostellar hooks are presented on the basis of bright-field microscopy, confocal laser scanning microscopy, Normaski differential interference contrast light microscopy and variable pressure scanning electron microscopy.

MATERIAL AND METHODS

The *E. granulosus* protoscoleces used in this study were obtained from livers and lungs cysts of ovines and bovines origin from abattoirs in Peruvian provinces Arequipa (16° 20' S and 71°30' W), Cuzco (13°32' S and 72°00' W), Puno (15°55' S and 70°03' W), Huancavelica (12° 50' S and 75° 05' W) and Junin (11° 24' S and 76° 00'W) (Almeida *et al.*, 2007).

Protoscoleces rostellar hooks were aspirated under sterile conditions together with the hydatid fluid from each cyst and rinsed twice in a 0.85% NaCl solution. Each suspension of protoscoleces was sieved to remove the larger debris (Ahmadi, 2004). Then, the material was transferred and stored in small tubes with 10% buffered formalin at room temperature.

Protoscoleces for bright-field microscopy (BM) were squashed under coverslip in polyvinyl lactophenol on microscope glass slides. Acquired images were obtained using Image Pro-Plus software (Media Cybernetics, Inc., Silver Spring, MD, USA). For confocal laser scanning microscopy (CLSM) whole-mounts were examined under a confocal microscopy (LSM 510 – ZETA, Zeiss) (Neves *et al.*, 2004). All of these slides were also examined under phase contrast by adding a prism (Zoom 3.8) to the scanning confocal microscope.

Nomarski differential interference contrast light microscope images of rostellar hooks were obtained from a Zeiss bright-field microscope in a differential interference contrast (DIC) apparatus.
For variable pressure scanning electron microscopy (VPSEM), hooks were rinsed in Milli-Q water followed by repeated centrifugation at 1000g for 2 min. Samples were placed on glass slides, and inspected under a LEO 435 VP SEM operating at 15000 V and 150 Pa. Several linear measurements and area of both large and small hook were analyzed: total area (TA), total perimeter (TP), total length (TL), total width (TW) and distance between blade and guard (BGD).
For the statistical analysis, all procedures were

carried out using Statistical Package for Social Sciences (SPSS version 9.0, Chicago). Statistical analysis included Analysis of variance (ANOVA) followed by the post-hoc Tukey-test. The statistical significance was assessed at $p<0.05$.

RESULTS

All microscopic techniques utilized evidenced morphologic variations in both large and small rostellar hooks, even though all of them presented handle, blade and guard (Fig. 1a). Large and small hooks presented a central amorphous pulp region (Fig. 1b). Large hooks (Figs. 1b-d) frequently had thin guards and an irregular surface between the guard and handle (Fig. 1c). There are some hooks with stout guards and smooth surface between the guard and handle (Figs. 1b-d). Small hooks (Figs. 1e-f) presented rounded and stout guards (Figs. 1e-f). Usually, the surface between the guard and handle was irregular (Fig. 1a). The blade did not show any relevant feature.

We obtained good results with differential interference contrast microscopy (DIC). Some protoscoleces settled in the cyst fluid (hydatid sand) were joined by a slender stalk (Fig. 2a). They possess a variable number of rostellar hooks arranged in two rows (Fig. 2 b), alternating between large (upper row) and small hooks (lower row).

It was possible to remove a small number of hooks from the rostellar pad and analyze the marks they left on the rostellum. Variable pressure scanning electron microscopy (VPSEM) imaging revealed that the marks left by the missing upper row hooks were larger than lower row ones (Fig. 2c). The larger hooks are located in the upper row (Fig. 2d).

Confocal laser scanning microscopy (CLSM) images confirmed that large hooks had thin guards with an irregular surface between the guard and handle (Fig. 3a). Hooks with stout guards and a smooth surface between the guard and handle was found (Fig. 3b). Small hooks also presented rounded and stout guards (Fig. 3c), and usually the surface between the guard and handle was irregular (Fig. 3d).

The morphometric approach allowed discriminating differences between large and small hooks. The hooks of the upper row were found to be significantly longer than those of the lower row. The average measurements of large hooks were 101µ m^2 (TA), 61µm (TP), 25µm (TL), 9µm (TW) and 10µm (BGD). Regarding the small hooks, the average measurements of were 79µm (TA), 52µm (TP), 22µm (TL), 8µm (TW) and 10µm (BGD).

DISCUSSION

Many issues concerning the taxonomy of *E. granulosus* have been resolved on the basis of morphological and morphometric techniques. For these purposes, there is often a need to collect images for recording metacestode rostellar hooks. To date, only conventional morphological techniques as bright-field microscopy, scanning electron microscopy and transmission electron microscopy were used (Kumaratilake *et al.*, 1986; Smith & Richards, 1991; Antoniou & Tselentis, 1993; Dubinský *et al.*, 1998; Xiao *et al.*, 2005). The potential value of new techniques does not seem to have been deeply realized for *E. granulosus* studies.

Usually, for bright-field analysis unstained mounts of hydatid fluid sediment are examined due to difficulty of staining rostellar hooks (Clavel *et al.*, 1999). In this work, the features and distribution of unstained rostellar hooks from Peruvian samples under several morphological techniques were examined. This study and others (Said *et al.*, 1988; Hobbs *et al.*, 1990; Ponce-Gordo & Cuesta-Bandera, 1997; Dubinský *et al.*, 1998; Turcekov? *et al.*, 2003; Ahmadi, 2004; Thompson *et al.*, 2006) evidenced a high degree of polymorphism within hooks. We observed that large hooks had a slender guard, whereas the guard of the small hooks was more robust and rounded. The region between the guard and the handle was serrated. In contrast, scanning electron studies revealed that large hooks were longer, more pointed, slightly curved and less robust in comparison with small hooks. The large hooks had a projecting region with a smooth surface, whereas small hooks had a serrated edge (Antoniou & Tselentis, 1993).

During protoscolex development this parasitic form remains attached to the germinative layer through a stalk. When fully differentiated, the stalk is cut off and the infective protoscolex is now free in the hydatid fluid (Galindo *et al.*, 2002). On observing

brood capsules under bright-field microscopy, our data confirm that developing stages already harbor rostellar hooks, which are the first fully, differentiated structures formed at the apical region of the nascent scolex (Galindo *et al.*, 2002).

The major question arising from these findings is that large and small hooks are not accurately discriminated on the basis of morphological tool, as recently observed for *Echinococcus shiquicus* (Xiao *et al.*, 2005). From a taxonomic viewpoint, the larval-hook morphometry is a valid method for identifying *E. granulosus* (Ahmadi, 2004). The results obtained here indicate that large and small hooks can be reliably distinguished on the basis of morphometric differences between them. This result agrees with previous studies (Ponce-Gordo & Cuesta-Bandera, 1997; Ahmadi, 2004; Almeida *et al.*, 2007).

An important finding in relation to previous studies (Antoniou & Tselentis, 1993; Xiao *et al.*, 2005) refers to the two rows of rostellar hooks: large (upper row) and small hooks (lower row). Because no specific sample preparation protocols are required, variable pressure scanning electron microscopy (VPSEM) now is widely used for morphological studies (Griffin, 2007). This technique was chosen because does not requires some proceeding that makes possible damages to the preparation. This approach combines the advantages of light microscopy and resolution of electron microscopy (Muscariello *et al.*, 2005). Variable pressure scanning electron microscopy imaging revealed that the marks left by the missing upper row hooks were larger than lower row ones. In addition, larger hooks are located in the upper row. Records exist indicating that these differences in the morphology of the two types of hooks are related to the differences in their function (Antoniou & Tselentis, 1993).

Confocal imaging has been demonstrated both theoretically and practically to give improved visibility of optical sections and improved resolution. Since the images produced by the confocal system are held in a digital frame store they are readily available for computer analysis (White *et al.*, 1987). Another advantage is that a same whole-unt can be observed either by BM or CLSM. Our previous studies evidenced that CLSM allows the study of gross anatomy in chloride carmine-stained helminthes (Machado-Silva *et al.*, 1998; Neves *et al.*, 2004). However, in this study the rostellar hooks failed to stain with chloride carmine (data not shown). In this respect, CLSM images of unstained hooks were obtained with a prism coupled for the phase contrast technique. Apart from their low degree of fluorescence, the protocol followed in this study allowed us to support the notion that rostellar hooks show morphological polymorphism, as above described for bright-field analysis. Consequently, it is still difficult to accurately discriminate between these two rostellar hooks on because morphological variations were evidenced. Finally, future studies based on stained rostellar hooks are needed (Clavel *et al.*, 1999).

In conclusion, the current data show that large and small rostellar hooks have morphological polymorphism. Because the application of this knowledge for taxonomic study is limited, for this end morphometry techniques are required. Our study demonstrated the usefulness of combining conventional and new morphological tools to help to solve unresolved matters with regards to rostellar hooks features.

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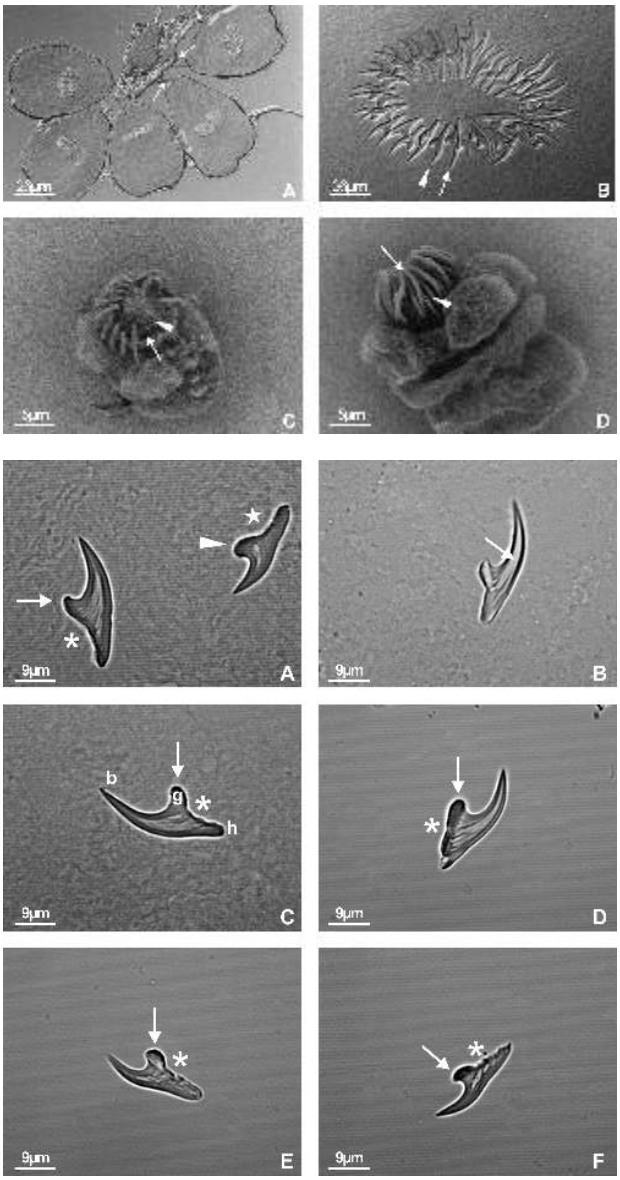

Figure 1. Bright-field micrographs of *Echinococcus granulosus* large and small rostellar hooks. **a** Large (left) and small (right) rostellar hooks showing the guard (arrows) and the surface between the guard and handle (⁋, *), **b** large hooks showing the central amorphous pulp (arrow), **c - f** rostellar hooks showing the guard (arrows) and the surface between the guard and handle (*⁋): b, blade; g, guard; h, handle. Scale bar, 9µm.

Figure 2. Photomicrographies of *Echinococcus granulosus* metacestodes. **a** Bright-field images in a differential interference contrast of brood capsules containing protoscoleces joined by a slender stalk (arrow), bar 26µm; **b** Bright-field images in a differential interference contrast of large (→) and small (▲) rostellar hooks, bar 26µm; **c** Variable pressure scanning electron microscopy micrographs showing marks left by the missing large (→) and small hooks (▲), bar 5µm; **d** Variable pressure scanning electron microscopy micrographs showing rostellar hooks showing large hooks in the upper row (→) and small hooks in lower row (▲), bar 5µm.

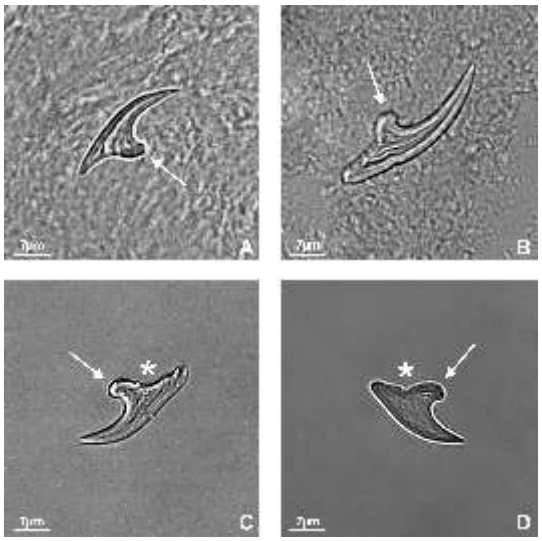

Figure 3. Confocal laser scanning images of *Echinococcus granulosus* showing rostellar hooks. **a - b** Large rostellar hooks showing the guard (arrows), **c - d** Small rostellar hooks showing the guard (arrows) and the surface between the guard and handle (⁋). Scale bar, 7µm.

BIBLIOGRAPHIC REFERENCES

Ahmadi, NA. 2004. *Using morphometry of the larval rostellar hooks to distinguish Iranian strains of* Echinococcus granulosus. Annals of Tropical Medicine and Parasitology, vol. 98, pp.211-220.

Ahmadi, N & Dalimi, A. 2006. *Characterization of* Echinococcus granulosus *isolates from human, sheep and camel in Iran.* Infection, Genetics and Evolution, vol. 6, pp.85-90.

Almeida, FB, Rodrigues-Silva, R, Neves, RH, Romani, EL & Machado-Silva, JR. 2007. *Intraspecific variation of* Echinococcus granulosus *in livestock from Peru.* Veterinary Parasitology, vol.143, pp.50-58.

Antoniou, M & Tselentis, Y. 1993. *Studies on* Echinococcus granulosus *using the scanning electron microscope. II. The hooks*. Parasitology Research, vol.79, pp.543-546.

Clavel, A, Varea, M, Doiz, O, Lopez, L, Quilez, J, Castillo, FJ, Rubio, C & Gomez-Lus, R. 1999. *Visualization of hydatid elements: comparison of several techniques.* Journal of Clinical Microbiology, vol.37, pp.1561-1563.

Dubinský, P, Stefancì ková, A, Turceková, L, Macko, JK & Soltýs, J. 1998. *Development and morphological variability of* Echinococcus granulosus. Parasitology Research, vol.84, pp.221-229.

Eckert, J & Deplazes, P. 2004. *Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern.* Clinical Microbiology Reviews, vol.17, pp.107-135.

Galindo, M, Gonzalez, MJ & Galanti, N. 2002. Echinococcus granulosus *protoscolex formation in natural infections.* Biomedical Research, vol.35, pp.365-371.

Griffin, BJ. 2007. *Variable pressure and environmental scanning electron microscopy: imaging of biological samples.* Methods in Molecular Biology, vol.369, pp. 467-495.

Hobbs, RP, Lymbery, AJ & Thompson, RC. 1990. *Rostellar hook morphology of* Echinococcus granulosus *(Batsch, 1786) from natural and experimental Australian hosts, and its implications for strain recognition*. Parasitology, vol.101, pp.273-281.

Ingold, K, Dai, W, Rausch, RL, Gottstein, B & Hemphill, A. 2001. *Characterization of the laminated layer of in vitro cultivated* Echinococcus vogeli *metacestodes.* Journal of Parasitology, vol.87, pp.55-64.

Kumaratilake, LM, Thompson, RCA & Eckert, J. 1986. Echinococcus granulosus *of equine origin from different countries possess uniform morphological characteristics*. International Journal of Parasitology, vol.16, pp.529-540.

Machado-Silva, JR, Pelajo-Machado, M, Lenzi, HL & Gomes, DC. 1998. *Morphologic study of adult male Worms of* Schistosoma mansoni *Sambon, 1907 by confocal laser scanning microscopy.* Memûias do Instituto Oswaldo Cruz, vol. 93, pp.303-307.

Moro, PL, Lopera, L, Cabrera, M, Cabrera, G, Silva, B, Gilman, R & Moro, MH. 2004. *Short report: endemic focus of cystic echinococcosis in a coastal city of Peru.* American Journal of Tropical Medicine and Hygiene, vol.71, pp.327-329.

Muscariello, L, Rosso, F, Marino, G, Giordano, A, Barbarisi, M, Cafiero, G & Barbarisi, A. 2005. *A critical overview of ESEM applications in the biological field.* Journal of Cellular Physiology, vol.205, pp.328-334.

Neves, RH, Costa-Silva, M, Martinez, EM, Branquinho, TB, Oliveira, RMF, Lenzi, HL, Gomes, DC & Machado-Silva, JR. 2004. *Phenotypic plasticity in adult worms of* Schistosoma mansoni *(Trematoda: Schistosomatidae) evidenced by bright field and confocal laser scanning microscopies.* Memûias do Instituto Oswaldo Cruz, vol. 99, pp.131-136.

Ponce-Gordo, F & Cuesta-Bandera, C. 1997. *Differentiation of Spanish strains of* Echinococcus granulosus *using larval rostellar hook morphometry.* International Journal for Parasitology, vol.27, pp.41-49.

Rogan, MT & Richards, KS. 1987. Echinococcus granulosus: *changes in the surface ultrastructure during protoscolex formation.* Parasitology, vol.94, pp.359-367.

Said, IM, Abdel-Hafez, SK & Al-Yaman, FM. 1988. *Morphological variation of* Echinococcus granulosus *protoscoleces from hydatid cysts of human and various domestic animals in Jordan.* International Journal for Parasitology, vol.18, pp.1111-1114.

Smith, SA & Richards, KS. 1991. *Ultrastructure and microanalyses of the protoscolex hooks of* Echinococcus granulosus. Parasitology, vol.103, pp.267-274.

Tashani, OA, Zhang, LH, Boufana, B, Jegi, A & McManus, DP. 2002. *Epidemiology and strain characteristics of* Echinococcus granulosus *in the Benghazi area of eastern Libya.* Annals of Tropical Medicine and Parasitology, vol.96, pp.369-381.

Thompson, RCA, Boxell, AC, Ralston, BJ, Constantine, CC, Hobbs, RP, Shury, T & Olson, ME. 2006. *Molecular and morphological characterization of* Echinococcus *in cervids from North America.* Parasitology, vol.132, pp.439-447.

Thompson, RCA & McManus, DP. 2002. *Towards a taxonomic revision of the genus* Echinococcus. Trends in Parasitology, vol.18, pp.452-457.

Turčekovl, L, Snábel, V, D'Amelio, S, Busi, M & Dubinský, P. 2003. *Morphological and genetic characterization of* Echinococcus granulosus *in the Slovak Republic.* Acta Tropica, vol.85, pp.223-229.

White, JG, Amos, WB & Fordham, M. 1987. *An evaluation of confocal versus conventional maging of biological structures by fluorescence light microscopy.* The Journal of Cell Biology, vol.105, pp.41-48.

Xiao, N, Qiu, J, Nakao, M, Li, T, Yang, W, Chen, X, Schantz, PM, Craig, PS & Ito, A. 2005. Echinococcus shiquicus *n. sp., a taeniid cestode from Tibetan fox and plateau pika in China*. International Journal for Parasitology, vol.35, pp.693-701.

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