Research Square

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Protoscolex evagination and pre-worm maintenance with bile are key processes for adult worm development of Echinococcus granulosus and Echinococcus multilocularis in vitro

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Research Article

Keywords: Echinococcus granulosus, Echinococcus multilocularis, protoscoleces, evagination, adult worm development, bile salts

Posted Date: November 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2208857/v1

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Abstract

Background: In vitro cultivation of *Echinococcus* is essential for vaccine development to prevent transmission of echinococcosis to dogs. We optimized the conditions for *Echinococcus*protoscolex evagination and adult worm development in vitro, including those of water, bile, bile salt, trypsin, and serum in the culture system.

Results: Short stimulation (2 × 20 s) with water significantly increased the evagination of protoscoleces (pre-worms) of both *E. granulosus* and *E. multilocularis*. However, medium containing fetal calf serum (FCS) invaginated 92% of these evaginated protoscoleces. Preculture of the evaginated protoscoleces in no-serum RPMI1640 medium containing dog bile or bile salt for three days maintained 80.5% of the evaginated protoscoleces. Dog serum gel-base maintained 79.8% of the evaginated protoscoleces developing adult worms, which was higher than newborn bovine serum gel-base. The rapidly developing worms had 3–4 progloids after 56 days of culture. *E. granulosus* worms were longer and wider in size than *E. multilocularis* after five weeks of in vitro culture.

Conclusions: Brief stimulation with water for proscolex evagination and pre-worm maintenance in no-serum medium are crucial for in vitro worm development of *E. granulosus* and *E. multilocularis*. Dog serum gel-base and bile salts are important for long-term tapeworm development.

This study was supported by the National Natural Science Foundation of China (81830066, U1803282)

Background

Echinococcosis is a widespread zoonotic disease that includes cystic echinococcosis (CE) caused by the dog tapeworm *Echinococcus granulosus sensu stricto* and alveolar echinococcosis (AE) caused by the fox tapeworm *Echinococcus multilocularis*. The high endemic areas of the disease are Central Asia—including western China, North Africa, and South America [1, 2, 3, 4]. *Echinococcus* is a small tapeworm that resides in the intestine of carnivore hosts[5]. Both *E. granulosus ss* and *E. multilocularis* require two mammalian hosts including definitive hosts (dogs, foxes, and wolves) and intermediate hosts (sheep, cattle, and small mammals) to complete their life cycle [5]. Dogs or other carnivore animals swallow the grazing animal offal, such as liver or lungs, containing echinococcal protoscoleces. These small larvae develop to adult worms in the intestine of the definitive animals. After 45 d for *E. granulosus* and 30 d for *E. multilocularis*, eggs (from gravid proglotitids) are produced and released through the host’s feces to the environment[5]. The eggs are ingested by an intermediate host in which the metacestode (cysts or vesicles) and protoscoleces are developed. The cycle is completed if the protoscoleces are eaten by a suitable carnivore.

Both CE and AE are chronic parasitic diseases that can remain asymptomatic in humans for up to 15 years [6]. Control of CE in New Zealand has been achieved by dosing dogs every 45 d [7]. However, it is difficult to control these diseases in countries in large continents. In addition, there are no successful control measures for AE, as its sylvatic life-cycle involves wild carnivores (mainly red fox, *Vulpes vulpes*,

and arctic fox, *Alopex lagopis*) as its most important definitive hosts. Small mammals (usually microtine and arvicolid rodents) that serve as intermediate hosts are extremely difficult to control[3, 7]. New measures are needed to effectively control these diseases.

Dogs are pivotal in *Echinococcus* transmission, especially to humans. Dog vaccination is a practical and cost-effective prevention strategy, because there are far fewer dogs than sheep in the endemic areas and fewer animals need to be vaccinated [8]. However, the development of a dog vaccine against *Echinococcus* has been difficult, and more biological studies on the tapeworms are needed. Despite extensive morphological study, little is known about the molecular biology associated with the developmental process of the tapeworms including attachment to the intestine wall of dogs, growth, proglottisation, maturation, and sexual development. An in vitro cultivation model is essential for conducting these biological studies. Smyth previously achieved successful adult culture in vitro [8,[9]. We were able to repeat his cultivation methods, but were unable to obtain a high rate of evaginated protoscoleces and adult worm development.A

Smyth noted that many factors impact the evagination of protoscoleces and adult worm development in vitro[9] In this study, we optimized the conditions for protoscolex evagination and adult worm development in vitro, including dog bile, bile salt, trypsin, water, and serum in the medium for maintaining the evaginated protoscoleces and optimizing adult worm growth. The successful in vitro culture of adult *E. granulosus* and *E. multilocularis* worms provides a platform for investigating the biology of adult worm development and screening effective therapeutic drugs against these two tapeworm species.

Materials And Methods

Animals

Three female beagle dogs (12 months of age) were purchased from the Animal Center of Xinjiang Medical University for collecting serum and Gerbils (*Meriones unguiculatus*) (6–8 weeks of age) for maintaining *E. multilocularis* were purchased from Beijing Vital River Laboratory Animal Technology Company Ltd. The animals were housed in specific pathogen-free (SPF) facilities at the First Affiliated Hospital of Xinjiang Medical University (FAH-XMU). The use of these animals was approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University (approval IACUC-2015 and IACUC-2013).

Parasites

Protoscolex collection

Sheep livers containing *E. granulosus sensu stricto* cysts were collected from a slaughterhouse in Urumqi, Xinjiang, China. Collection of protoscoleces (PSCs) from the livers was accomplished using previously published methods[10].

E. multilocularis PSCs were collected from the abdominal cavity of infected gerbils as previously described[11]. PSCs of *E. granulosus* and *E. multilocularis* were rinsed with phosphate buffer solution (PBS) three times by natural precipitation. One milliliter of precipitated PSCs were digested with 20 ml of 1% of pepsin (Sigma, St. Louis, MO) freshly prepared with Hanks solution, pH2.0. After 30 min, the digested PSCs were rinsed five times with PBS. The viability of the PSCs was determined by 0.1% methylene blue. PSCs with viability > 98% were retained for further use.

Options For Evagination And Cultivation Of Protoscoleces

To increase evagination of PSCs in vitro, a short period of water stimulation was used for PSC pre-treatment. The pepsin digested PSCs were immersed in 1 mL of water for 20 s and then immediately diluted with 10 mL of PBS. Two repetitions of this process stimulated evagination of 90–100% of the PSCs. We used these evaginated PSCs to study the maintenance options of evaginated PSCs or pre-worms.

The evaginated PSCs were initially cultured in 30 mm culture dishes with each well containing 1000–1500 PSCs in 2 ml RPMI-1640 or PBS medium. Sixteen groups were designed as shown in Table S2 with three replicates for each treatment. The optional stimulation conditions included different concentrations of dog bile (0.01–0.05%), bile salts (T4009, Sigma, Louis, MO) (0.0002–0.001%), and trypsin (0.1–0.5%) in PBS or RPMI-1640 medium. The evagination rate of protoscoleces was observed after 48 h of cultivation.

Long-term Adult Worm Development

Since trypsin may play an important role in evagination of PSCs, different concentrations of trypsin (0.1–0.5%) were used to determine the optimal level. PSCs were divided into five groups (Groups 1–5) and precultured in RPMI-1640 without bovine serum for 3 d. They were then cultured in a medium containing 20% fetal bovine serum for 14 d.

To confirm the role of dog bile in long-term adult worm cultivation, the water-stimulated PSCs were divided into another five groups with dog bile (0.01–0.05%) (Groups 6–10 in Table S3). The development of adult worms was observed after 12 h, 48 h, 4 d, 7 d, 10 d, and 14 d. Three replicates were established for each test. One blank control was untreated.

Combination Effect Of Dog Bile And Trypsin In Maintenance Of Pre-worm Cultivation

To optimize the co-effect of dog bile and trypsin stimulation on the evagination maintenance of PSCs, the water treated PSCs were divided into 16 groups with each well containing 1500 PSCs in 2 mL RPMI-1640 medium (Table S3). Three replicates were set up for the optional test. The culture conditions were 38.5°C,

5% CO_2, and 100% humidity, and the medium was replaced with fresh medium every 3 d. The samples were collected and observed with light microscope to count the evagination and activation of the PSCs at 12 h, 48 h, 4 d, 7 d, 10 d, and 14 d after cultivation.

Adult Worm Culture

The full culture medium was prepared according to the recipe of Smyth with RPMI-1640 (Hyclone) instead of M199 (Table S1) and containing 20% fetal bovine serum (Hyclone), 0.4% yeast extract (Sigma, USA), 0.4% glucose (Sigma, USA), and 100 μG/ml double antibody (Hyclone) filtered through a 0.22-μM filter. Fresh dog bile was collected from culled stray dogs in echinococcosis control areas and diluted to 20% (v/v) with normal saline. The diluted bile was stored at − 20ºC after it was filtered through a 0.22-μM filter. Trypsin (Sigma, USA, 10%) was diluted with either saline or culture medium.

To optimize the growth conditions, we used the Smyth worm culture system containing two liquid media and a solid phase [9, 12]. The solid phase was made by either newborn bovine serum or beagle dog serum. For preparation of the serum gel-base, 20 ml of newborn bovine serum or beagle dog serum was added to a 100-ml glass bottle and then heated at 95°C for 40 min in a manner similar to that used by Smyth [9].

The culture condition was same as above and worm development was observed at 21 d, 35 d, 45 d, and 56 d post culture. We also studied the long-term culture using, or without using, dog serum gel-base, with or without dog bile.

Statistical analysis

Statistical Product and Service Solutions (SPSS 26.0) was used for statistical analysis of the data. Measurement data are expressed as mean ± standard deviation (x − ±SD). Chi-squared test was used to compare two groups and analysis of variance (ANOVA) was used for comparisons between multiple groups. Fisher’s least significant difference test (LSD) was used to test the difference between two groups. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of water and dog bile on evagination of protoscoleces (PSCs)

To increase the evagination of PSCs, we stimulated PSCs with water in a range of options including different time(from 10 s to 60 s) per stimulation (data not shown). The best option identified was stimulating PSCs two times with water with each soaking the PSCs for 20 s, and then equilibrated with 10 times the volume of normal saline. Using this method, 85%–90% of the PSCs were evaginated (Fig. 1a). After evagination, the PSCs, especially their suckers, were very active.

Our next step was to maintain these evaginated PSCs. However, when we added these evaginated PSCs into RPMI 1640 containing 10%–20% serum, 61% of the PSCs invaginated following 12 h culture. We then cultured the evaginated PSCs in RPMI1640 or PBS without bovine serum for 3 d, which kept 82.7% of PSCs evaginated (Fig. 1b).

When adult *E. granulosus* and *E. multilocularis* worms grow in the intestines of dogs, the gastrointestinal solution or mucosa contains dog bile and trypsin. Therefore, the third step was to culture these pre-worms with different concentrations of dog bile or bile salt in the medium. We added 0.01%–0.05% of dog bile to PBS or RPMI-1640 medium without serum and found that 0.02% of dog bile was the best concentration for maintaining the evaginated PSCs. After 48 h of culture, the evagination rate of PSCs was the highest in both cultures, with 48.4% in PBS and 80.9% in RPMI-1640 medium. Both were significantly higher than that in PBS ($P < 0.05$). When the dog bile level was increased to 0.04%, the evagination rate of PSCs decreased to 56.2%.

We also determined that 0.0004% of bile salt (T4009) in RPMI-1640 medium maintained 79.1% evaginated PSCs, which was lower than that of 0.02% of dog bile ($P < 0.05$), but higher than 0.04% of dog bile ($P < 0.05$), as shown in (Fig. 1c 1d). Therefore, RPMI-1640 medium supplemented with 0.02% dog bile was used in the subsequent adult culture experiment.

Effects of trypsin in maintenance of evaginated protoscoleces and pre-worm culture

Since trypsin is also a key compound in the intestine of dogs, we used different concentrations of trypsin (0.1%–0.4% w/v) in RPMI1640 medium to study the impact on PSC evagination and pre-worm culture. After overnight (12 h) culture, trypsin showed little effect on the evagination rate of PSCs. However, 0.2% trypsin produced the highest proportions of evaginated PSCs at 2 d (52.7%), 4 d (82.1%), and 7 d (81.9%), respectively (Fig. 2). When the final concentration of trypsin increased to 0.4%, the evagination rate of PSCs decreased to 56.22% compared to 82.1% by 0.2% of trypsion after 4 d of culture($P<0.05$) (Fig. 2b). Thus, the ideal concentration of trypsin was 0.2% in RPMI-1640 medium.

After 48-h dog bile stimulation, the 0.02% concentration of dog bile showed the highest PSC evagination rate. The 14-d culture group confirmed that 0.02% bile was the best for worm culture (Fig. 2). After the final concentration of dog bile was set to 0.02%, the evaginated pre-worm were cultured in RPMI-1640 medium for 7 d, 10 d, and 14 d. The evagination rates were significantly higher than those of the other treatment, reaching 59.5%, 70.7%, and 79.7%, respectively ($P < 0.05$) (Fig. 2a).

Combination effect of dog bile and trypsin on protoscolex evagination and adult development

Since both dog bile and trypsin coexist in the dog intestine mucosa, we suspected a co-effect of these two chemicals on protoscolex evagination and adult development. We added different concentrations of trypsin (0.1%, 0.2%, and 0.3%) combined with two concentrations of dog bile, 0.01% and 0.02%, in RPMI-1640 medium for the first 3 d of pre-culture. Trypsin 0.2% with 0.02% of dog bile produced a significantly

higher number of evaginated PSCs and worms than that of other groups at 2 d, 7 d, 10 d, and 14 d, which were 81.7%, 67.5%, 71.4%, and 64.2%, respectively (*P<0.05*) (Fig. 3).

Key elements from the bidirectional development of *E. granulosus* and *E. multilocularis*

According to the cultivation method of Smyth, newborn bovine serum gel-base was important for the maintenance or long-term in vitro culture of adult worms. At 17–21 d post infection, the demarcation was formatted by an infolding of the tegument, indicating that segmentation or proglottisation occurs[5]. However, very few worms developed to the 3–4-segment stage. Therefore we used dog serum gel-base instead of gel-base made with newborn bovine serum. Dog serum gel-base increased the activity and adult worm growth of both *E. granulosus* and *E. multilocularis* (Fig. 5).

After 56 d, 38.7% of adult worms cultured with medium containing 0.02 dog bile on dog serum gel-base developed 4 segments, including a scolex head segment, neck proglottid, immature proglottid, and mature-proglottid (arrowed in Fig. 4c).

The parasites had two basic types of morphologies after 14 d of in vitro cultivation: evaginated PSCs or pre-worms and invaginated naive protoscoleces. Pre-worms had a smooth edge with clearly distinct internal and external structures, including a rostellum, hooks, and suckers (arrowed in Fig. 4a). Protoscoleces were invIginated with their scolex, rostellum, and suckers curled back.

After 56 d of bile-free cultivation, a majority of the invaginated PSCs or evaginated PSCs had developed into microvesicles and cysts, indicating that dog bile is a key element for adult worm development. In the cyst development, the scolex or head of protoscolex was expanded into a swollen shape, and the hooks and suckers gradually disappeared (Fig. 4b).

After 14 d of cultivation, a high evagination rate (78%) of protoscoleces was obtained in the RPMI1640 medium containing 0.02% bile cultured on the dog serum gel-base, with 5.4% mortality. After 21 d of incubation, PSCs began to strobilate the first segment of the adult worm. A total of 61% of the pre-worms had one budded segment. The mortality rate at this stage was 7.2% (Fig. 5d, Table 1). At the same time, the non-gel-base culture had 35% of PSCs evaginated, the mortality rate was 15.2%, and 34% of the PSCs developed into cysts (Fig. 5b). After 35 d of culture, 49% of worms developed 2–3 segments in the RPMI1640 medium containing 0.02% dog bile on the dog serum gel-base. At this stage, the mortality rate was 11.4%.

After 56 d of culture, 53% of adult worms had 3 or 4 segments. The mortality rate was 13.8%. However, this stage still had some PSCs that were in the invagnination stage. The percentages of worms with 1, 2, and 3 segments cultured by adults are shown in Fig. 5d and Table 1. However, in the bile free culture without gel-base to 56 d, more than 60% of the PSCs became cysts. Only 5% of the protoscoleces developed into 1-segment adults, and there were no other stages or worms (Fig. 5a).

Long-term development of adult *E. granulosus* and *E. multilocularis* in vitro

Based on the above options, we composed an optional culture system for in vitro adult worm development including evagination with two short periods (2 × 20 s) of water stimulation, maintenance incubation of the evaginated PSCs in non-serum RPMI1640 containing 0.02% dog bile and 0.2% trypsin for 3 d, then transferring these pre-tapeworms into a bottle with a dog serum gel-base containing RPMI1640 with 20% fetal calf serum, 0.02% dog bile, and 0.2% trypsin incubated at 38°C. This complete medium was more suitable for the growth of both *E. granulosus* and *E. multilocularis*. In the culture system, *E. granulosus* produced the first segment at 17–21 d post cultivation, the 2nd and 3rd segments at 25–30 d, 4th segment at 35–45 d, and the 5th segment at 50–55 d of cultivation. *E. multilocularis* adult development was faster than *E. granulosus* with one segment appearing at 15–21 d of culture, 2nd–3rd segments appeared at 22–28 d, and the 4th–5th segments at 32–45 d of cultivation.

The worm length of *E. granulosus* having 3 segments was 2.39 ± 0.12 mm (2.20–2.54 mm) on average, which was longer than *E. multilocularis*, with an average of 1.66 ± 0.10 mm (1.60–1.79 mm). When the worms had 4 segments, *E. granulosus* was 3.12±0.17 mm (2.89–3.44 mm) on average, which was longer than *E. multilocularis*, with an average of 2.06 ± 0.11 mm (1.89–2.21 mm) (Fig. 5a, b, Table 1).

Discussion

Adult *Echinococcus* worms develop in dog intestines in several key steps or stages, including protoscolex activation by gastrointestinal solution, protoscolex evagination, scolex attachment to the intestine wall, body proglottisation, sexual organ development, and egg production [5]. These are complex processes, with the maturity of *Echinococcus* adult worms taking about 35 d for *E. multilocularis* and 45 d for *E. granulosus*[5]. It is difficult to mimic the whole progress in vitro even with more than 30 years of our cultivation experience. However, Smyth and others have done pioneer studies and developed useful in vitro systems for both larval and adult stages[9, 12, 13, 14]. The underlying principles of differentiation, host–parasite relationships, and evolutionary biology have also been studied[5, 15, 16, 17].

The first step for *Echinococcus* adult worm growth is evagination of PSCs, which is essential for developmental processing. The unevaginated or invaginated protoscoleces cannot develop to adult worms, and instead, they develop into bladder cysts. There are few studies on the evagination of *Echinococcus* protoscoleces. Smyth completed a series of in vitro studies and demonstrated that dog bile and trypsin were key elements for the evagination. He showed that the culture medium containing 0.02% of dog bile and 0.1% of trypsin produced 61–78% of evaginated protoscoleces [18]. We used similar protocols with only culture medium Parker 199 replaced by RPMI1640. However, we never found that more than 10% of protoscoleces had evaginated after overnight cultivation. We tested a wide range of stimuli and found that soaking in water for a brief period is ideal for the evagination of PSCs.

To determine the optimal water soaking time, PSCs were stimulated for different times including 10, 20, 30, 40, and 60 s with 1 mL of water and then we recovered the PSCs with 10 mL of saline. We found that PSCs died with soaking times exceeding 30 s. We then used 20 s to stimulate PSCs each time. This was repeated two times, thus stimulating evagination in 90–100% of the PSCs.

Our next step was to determine how to maintain the evaginated PSCs. We used Smyth's method of adding pepsin digested PSCs directly into full culture medium containing bovine serum and found that less than 10% of the PSCs were evaginated. We thought this might be due to the high concentration of bovine serum forcing the PSCs into an inevaginated state. We then cultured the PSCs in medium without bovine serum, but containing dog bile and trypsin. These conditions significantly maintained the evagnination and activity of the PSCs. Considering that the developing pre-worms need nutrition, we added bovine serum to the medium at 3 d after cultivation. By using this method, 80.7% of PSCs were in an evaginated state and became very active after 3 d of adding bovine serum up to 20%. These changes with the culture medium indicated that evagination of PSCs is likely a physical process that may be related to the serum added and more likely due to change of the solution osmotic pressure [13] given that a water soaking increased the PSC evagination and non-serum RPMI1640 medium increased the evaginated PSCs.

For this reason, we used PBS and RPMI-1640 as a relatively low osmotic pressure solution. After 48 h cultivation in RPMI-1640, 78% of the PSCs remained evaginated, whereas PSCs cultured in PBS had a lower evagination rate and were not active. Compared to PBS, the RPMI164 medium contains more amino acids as nutrition, although the medium also contains other elements such as growth factors. The results indicate that nutrients such as amino acids are important for maintenance of the evagination of PSCs. In experimental infection of dogs with PSCs, we found that *E. granulosus* worms were increased remarkably when dogs were fed pure meat sausage after orally taken PSCs. Supplementing the diet with amino acids in early PSC development may be important for *Echinococcus* adult worm development, even in dogs.

We also compared the newborn bovine serum and dog serum made gel-base. The results showed that dog serum made gel-base was better than newborn bovine serum made gel-base. Therefore, we established an in vitro culture system in which 79.8% of PSCs developed to adult worms. In terms of worm growth, the first and third proglottids were formed after 17 d and 45 d, respectively, in our cultivation system. These times were reduced compared to the Smyth system, which required 30 d and 60 d, respectively.

After 21 d of culture, the rate of 1 segment of adult worm cultured on the dog serum gel-base was 59.3%. However, at 21 d post cultivation, the rate of worms containing the first segment was 4.2% without gel-base. Most PSCs (85%) were dead after 45 d of culture. Therefore, in addition to maintaining an appropriate concentration of dog bile during the culture process, the dog serum gel-base is also important for adult development.

Instead of dog bile, we used taurocholate sodium (T4009, Sigma), which produced results similar to those of dog bile. Bile acids often exist in the form of sodium or potassium salts, which form bile acid salts. Taurocholate (TC) and glycocholate (GC) account for 80% of the bile salt components. In addition, there are glycine goose deoxycholate, glycine deoxycholate, taurine goose deoxycholate, and taurine deoxycholate. Dog bile consists of taurine bound cholic acid, deoxycholic acid, and chennodeoxycholic

acid, while fox bile has only taurine bound cholic acid and deoxycholic acid [13]. This study verified that the effect of taurocholate sodium stimulation on the evagination rate of protoscoleces was similar to that of dog bile stimulation, indicating that taurocholate sodium can replace dog bile and plays a key role in the evagination and maintenance of adult *Echinococcus* worm development.

One important process is maintaining the evaginated PSCs or pre-worms. However, these evaginated PSCs will develop into bladder cysts if bile is not present in the culture medium. This indicates that bile is involved in regulating *Echinococcus* PSCs bidirectional development. In the presence of bile, PSCs develop into adult worms, while in the absence of bile, PSCs develop into cysts.

We also demonstrated the role of trypsin in the maintenance of the evaginated PSCs. Since a high concentration and high activity of trypsin occurs in the small intestine of dogs, we used different concentrations of trypsin to stimulate and maintain adult evagination. The results suggest that *E. granulosus* has natural resistance to trypsin, but this resistance is limited. The results also showed that more than 0.4% trypsin interfered with worm development and damaged the worm body. The evagination rate had a linear growth trend between 0% and 0.2% trypsin concentrations, and it decreased when the trypsin concentration exceeded 0.2%.

There are few existing studies on adult development of *E. multilocularis*. We showed that the two worm species are similar in their culture conditions. *E. multilocularis* developed faster than *E. granulosus* and the fast developing *E. multilocularis* took 15 d to produce the first segment, whereas *E. granulosus* took 17 d to produce the first segment. However, we did not obtain any worms producing eggs.

At 17–21 d post infection, the demarcation was formatted by an infolding of the tegument, indicating that segmentation or proglottisation occurs [5, 19]. The bottom region of the scolex extended, and the infolding tegument formed a new segment. Many biological processes may occur in this 7–14-d period including sexual organ formation. A more detailed analysis of these biological events may facilitate the discovery of dog vaccine target molecules. It is difficult to use dogs as an in vivo model to study dog worm development due to the ethical issues and infection risks involved. By using this new in vitro model, we can address many biological processes such as worm organ formation, bidirectional development, bile salts, worm differentiation and segmentation, and sexual and asexual reproduction.

Conclusion

The evagination of *E. granulosus and E. multilocularis* protoscoleces was significantly increased by a short period of water stimulation. Preculture of the evaginated protoscoleces in non-serum RPMI1640 containing 0.02% dog bile or 0.0004% taurocholate salt was essential for maintaining activity of the pre-worms. Without adding bile or bile salt, the cultured worms developed into bladder cysts. Newborn bovine serum gel-base or dog serum gel-base are key elements for the cultivation of adult worms for extended periods.

Abbreviations

AE, alveolar echinococcosis; CE, cystic echinococcosis; PSCs, protoscoleces

Declarations

Acknowledgments

We thank Mr Tao Jiang and Mr Chun Zhang for raising experimental animals, including beagle dogs and rodent animals, and for maintaining the *E. multilocularis* lifecycle in the laboratory.

Funding

The work was supported by grants from the National Natural Science Foundation of China (81830066, U1803282).

Availability of data and material

Data supporting the conclusions of this article are included within the article.

Author's contributions

WBZ and JL conceived and designed the experiments; PM, YLZ and CCW performed the experiments; WJQ and MXT participated in animal model and sample collection; WBZ and PM wrote the manuscript; PM and WBZ prepared figures and tables. WBZ, LJ, and YLZ critically revised the manuscript. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was conducted in accordance with the Chinese Laboratory Animal Administration Act (1988), and the study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no. IACUC-2015).

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Tables

Table 1. Percentage of worms at different stage of segmentation

Cultured days	Species	Percentage of worms having segments (%)					Length (mm)
		S	S+1	S+2	S+3	S+4	Mean ± SD
14	E. g	100	0	0	0	0	0.21 ± 0.04
	E. m	100	0	0	0	0	0.20 ± 0.03
21	E. g	61	39	0	0	0	1.03 ± 0.07
	E. m	59	41	0	0	0	0.74 ± 0.05
35	E. g	22	49	29	0	0	1.89 ± 0.08
	E. m	16	38	46	0	0	1.39 ±0.06
45	E. g	8	16	29	42	5	2.39 ± 0.12
	E. m	5	8	11	32	41	1.66 ± 0.10
56	E. g	7	11	29	33	20	3.12 ± 0.17
	E. m	10	9	22	31	28	2.06 ± 0.11

*E. g, *Echinococcus granulosus ss*; E. m, *Echinococcus multilocularis*; S, scolex;

S+1~4: scolex + number of prologttids

Figures

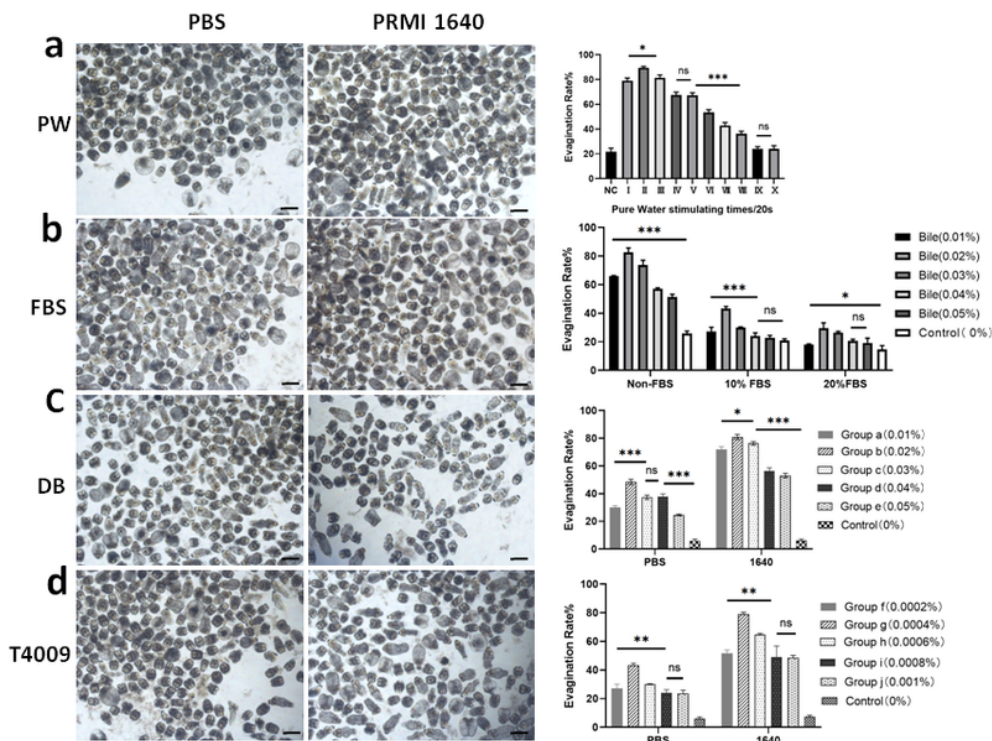

Figure 1

Figure 1

Cultivation of *Echinococcus granulosus ss* protoscoleces with different stimulators. a, initial stimulation with water (20 s/time). I–X indicates the number of stimulations from 1 to 10 times. b, after water stimulation, the PSCs were maintained in medium containing different percentage of fetal calf serum and different concentrations of dog bile. c and d, after water stimulation, the PSCs were maintained in solutions containing different concentrations of dog bile and bile salt T4009. PW, pure water; FBS, fetal bovine serum; DB, dog bile(0.02%); T4009, bile salt(0.0004%). Scale-bar: 100 μM.

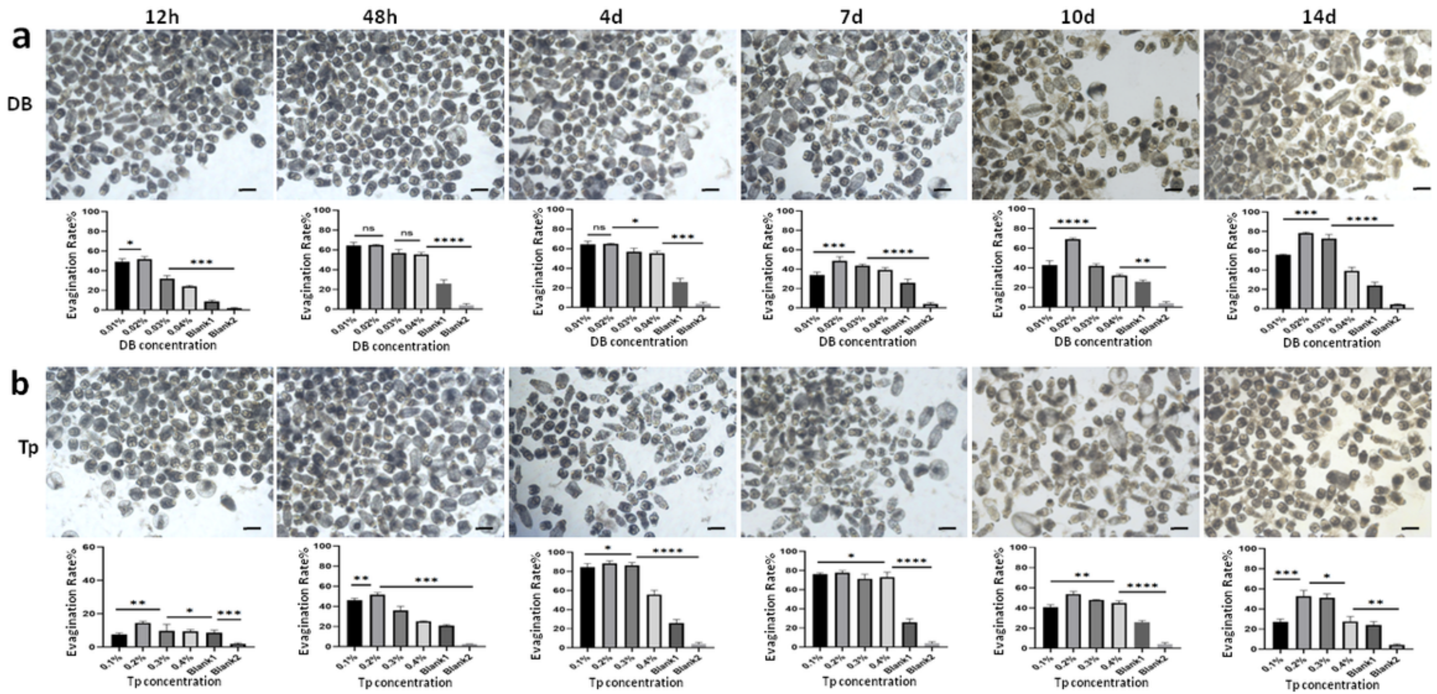

Figure 2

Impact of dog bile and trypsin on the preculture of evaginated protoscoleces. a and b. Dog bile (0.02%) and trypsin (0.2%) stimulation in 14 d of *E. granulosus*adult worm cultivation. Scale-bar: 100 μM.

Figure 3

Figure 3

Combination effect of dog bile and trypsin on development of *E. granulosus* adult worms for 14 d. DB, dog bile. Tp, trypsin. Scale-bar: 100 μM.

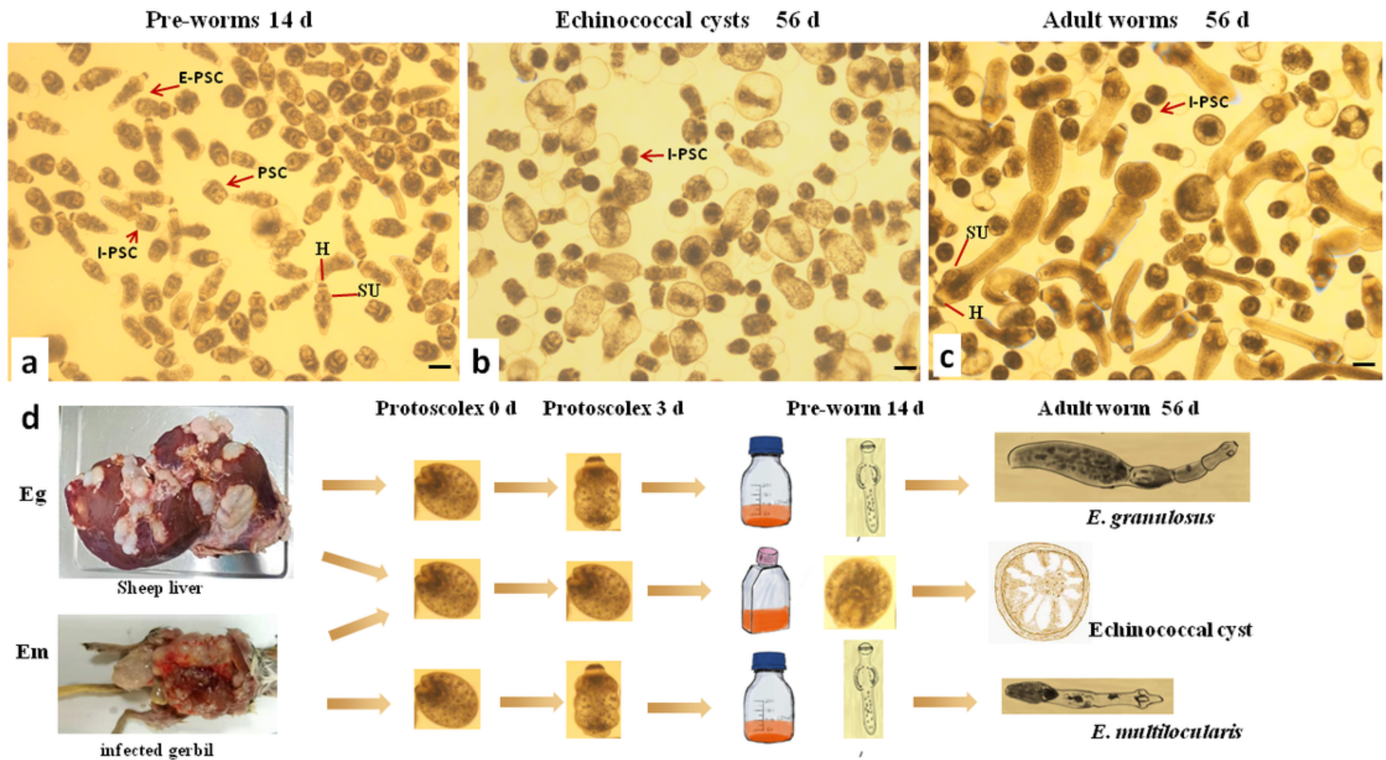

Figure 4

Morphological change of *E. granulosus ss* and *E. multilocularis* from protoscoleces to either metacestodes or adult tapeworms. a, b, and c, Development from 14 d to 56 d in vitro cultivation. d, Cultivation procedure in vitro. Abbreviations: PSC, protoscolex; SU, suckers; H, hooks; E-PSC, evaginated PSC; I-PSC, invaginated PSC. Scale-bar: 100 μM.

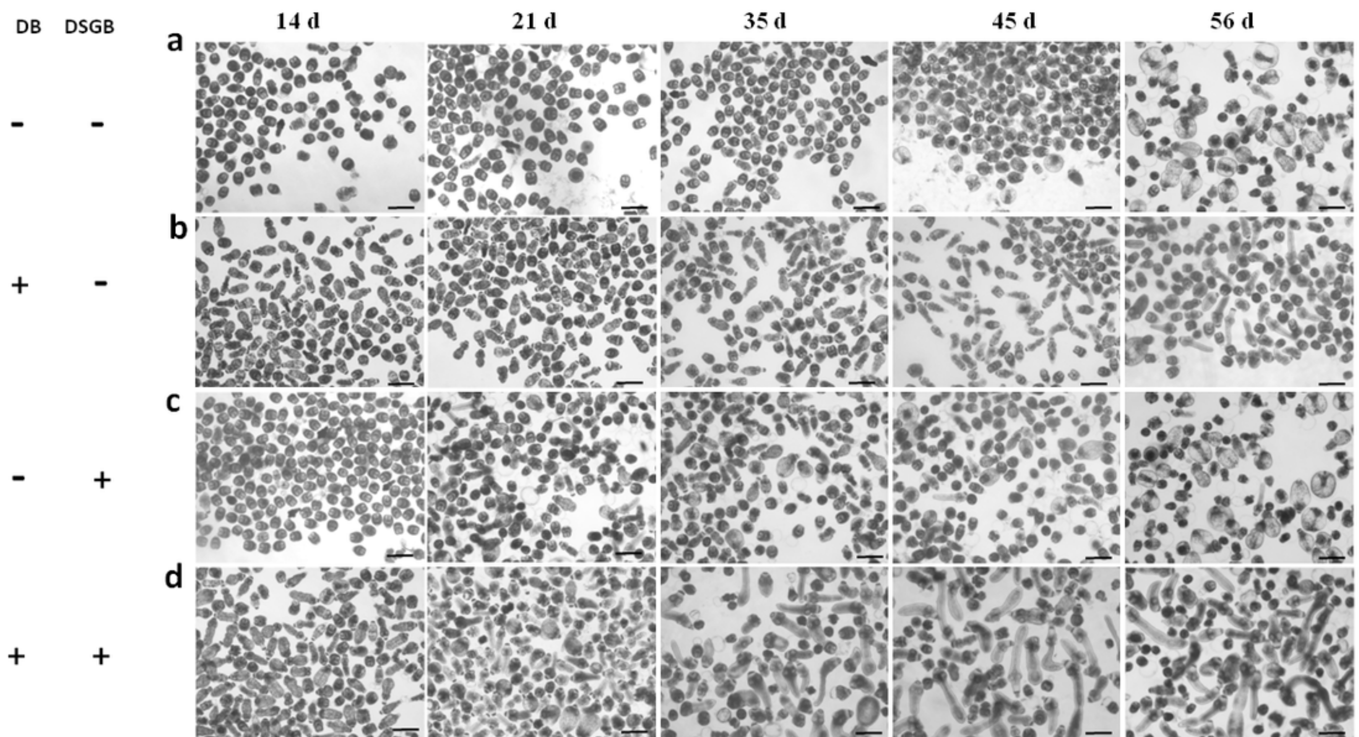

Figure 5

Dog bile and dog serum gel-base for long period culture of *E. granulosus ss* adult worms. a, in the medium containing no dog bile and without dog serum gel-base; b, in medium containing dog bile without dog serum gel-base; c, on the dog serum gel-based without dog bile in the medium; d, on dog serum gel-based with medium containing dog bile. DB, dog bile 0.02%; DSGB, dog serum gel-base. Scale-bar: 200 μM.

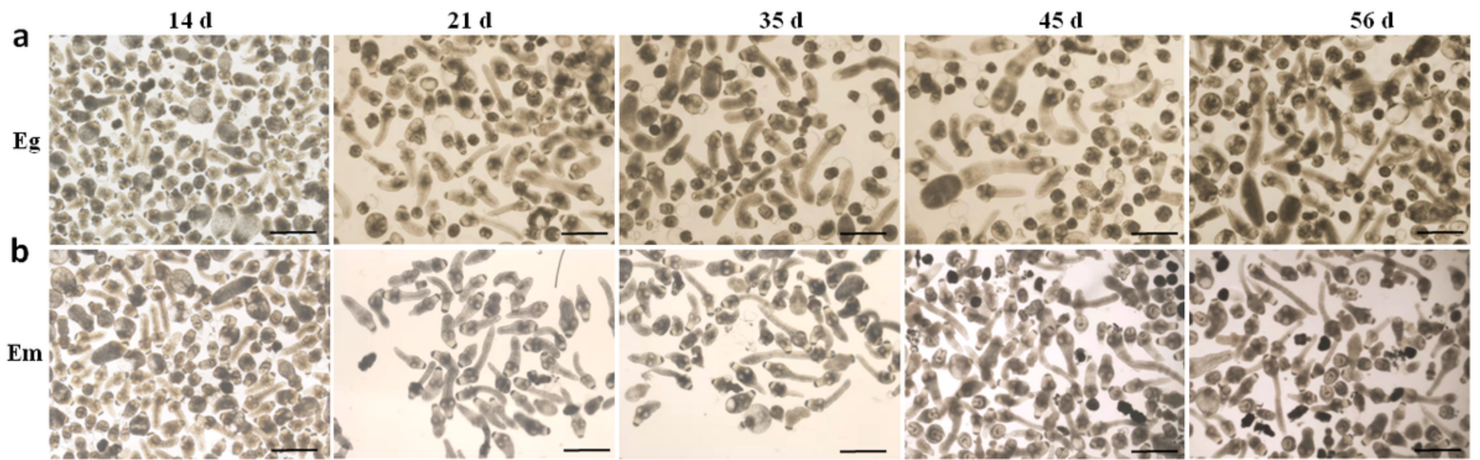

Figure 6

Figure 6

Cultivation of *E. granulosus ss* (Eg, a) and *E. multilocularis* (Em, b) in vitro. Scale-bar: 500 μM.

Supplementary Files

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- SupplementaryTables.docx