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Short Communication

Still and Moving Image Evidences for Mating of *Echinococcus granulosus* Reared in Culture Media

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Abstract

Background: *Echinococcus granulosus* cultivation is very important for improvement of different aspect of medical and veterinary researches. Despite many advances in this case, there is a missing link for in vitro life cycle of adult worms and it is fertilization. Regarding the researchers' observations, self-fertilization can be done in worms living in dog intestine, but despite all sorts of experimental techniques, this phenomenon has never been observed in reared worms in culture media. Furthermore cross fertilization has not been observed in vitro and even in parasites with dog intestinal origin; although it theoretically is possible. During a follow-up of cultivated adult worms, evidences of behaviors similar to self-mating (Type 2) and cross-mating were observed in our lab which will be presented here.

***Methods*:** Protoscoleces were aseptically removed from sheep hydatid cysts, washed twice with PBS and then cultivated in S.10E.H culture medium. The stages of parasite growth were observed using an inverted microscope for two months and all stages and behaviors were microscopically photographed. Different movies have also been made from these behavioral features.

***Results*:** After around 55 days post cultivation, some evidences of behaviors similar to self-mating (Type 2) and cross-mating were observed in some of the mature adult worms. However, fertile eggs in these parasites have never been observed.

***Conclusion*:** Regarding the above observations, these parasites show tendency to unsuccessful self-mating/fertilization (type 2) which failure could be due to anatomical position and physiological maturation. Also lack of suitable conditions for self-fertilization causes the worms try to do unsuccessful cross- mating/fertilization in culture media.

Introduction

Echinococcus granulosus causative agent of cystic echinococcosis is an important zoonotic worm with global distribution, hygiene and economic importance. The relative disease is prevalent in Middle East and many part of our country, Iran (1-4).

Cultivation of this parasite is very valuable for improvement of different branches of medico-veterinary researches. Although many advances have been done with cultivation methods, fertilization is a missing link for in vitro life cycle of *E. granulosus* (5-7). Regarding previous observations, self-fertilization is considered to be a normal way for sperm transfer in natural intestinal worms but despite several experimental techniques, it has never been seen in cultivated worms (6, 8). In addition, cross-fertilization has never seen even in intestinal parasite, although theoretically it is possible (8, 9) and rarely has been suggested by molecular studies (10, 11). During daily follow up of cultivated worms (12), we succeeded to detect some evidences of behavior similar to self-mating (type 2) and cross-mating which can be very important to further understanding of the biology and speciation of the parasite and subsequent researches. Here we will discuss our observations.

Materials and Methods

Hydatid cysts with more than 80% fertile protoscoleces (PSC) were collected from infected sheep at Shiraz abattoirs, cut opened under aseptic conditions, passed through two layers sterile gauze, washed three times with sterile PBS and then cultured in S10E.H culture media using the same procedures described before with some modifications (6,12,13). All stages of parasite growth were observed during a long term assay (about two months) from the first day of growth to adult forms, using an inverted microscope. Photos as well as movies were made during this follow up.

Results

After around 55 days post cultivation, 50-60% of initially PSCs were developed to adult worms with at least 3 proglottids in culture flasks. In addition, most of them had at least a mature segment. Active proglottids with protruded cirrus have frequently observed in mature adult worms. Genital pore also was opened and closed rhythmically special in latest segment of some of them. During daily control of the culture flasks we succeeded to detect some behavior similar to mating in reared *E. granulosus*. In a survey second segment of an individual worm connected to third one of another worm (Fig 1: A & B; Movie: 1). It was considered that the connection has occurred between cirrus of a worm and genital pore of another one. Furthermore in another observation an individual worm has attempted to close its third segment to the first segment of the same worm (Fig 1: C & D; Movie.2). Although these observations did not cause that we find mature eggs in culture flasks but these evidences may confirm positive potential of the *E.granulosus* to cross-mating/fertilization.

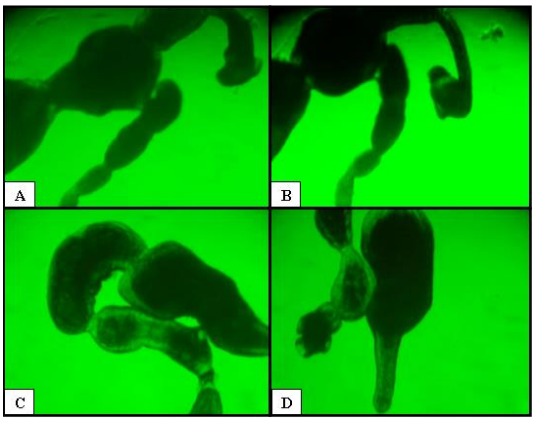

Fig.1: A and B- Connection between two different worms; C and D- Connection between first and third segment of an individual worm.

Discussion

Initial efforts were carried out since 1926 and 1928 by Dévé to grow PSCs in cyst fluid (14, 15). Smyth succeeded to introduce in vitro cultivation of different larval stages of this parasite (16). He also cultivated sexually mature strobilate of this parasite from PSC (5). Despite within several years many advances have been done with cultivation method but fertilization is missing link for in vitro life cycle of *E. granulosus* (5-7; 12-19; 21). *E. granulosus* is a hermaphroditic parasite. Presence of male and female reproductive systems and common genital pore shows the parasite has potential for both cross and self-fertilization (9). Probably self-fertilization is an advantage for this small worm that it might be difficult to find another worm particularly in light infections (22). This phenomenon has been observed in *E. granulosus* collected from naturally and experimentally infected definitive host (8, 9). In sections derived of dog worms, the cirrus was inserted into the vagina and morphologically has been proved seminal receptacle of intestinal worms filled with spermatozoa (8). Self-fertilization has also confirmed by some molecular studies in dog worms(10, 11, 23). Although cross-fertilization has not been observed in sections obtained of dog intestine (8) some researchers have suggested that aggregative behavior observed in intestinal worms are probably as a result of attraction between individual worms (24). Limited molecular reports have suggested probability of this phenomenon (10, 11) while others have rejected it (23). The major difference between sexually mature cultured and dog worms, was the failure of insemination to occur in the reared worms in culture media (25). In the well-developed genitalia of the mature reared adult worm many spermatozoa were seen in testes, but uterus filled with immature ova and empty seminal receptacle has always been observed (6).

In present study, we have frequently observed active proglottids with protruded cirrus. Genital pore was also opened and closed rhythmically special in latest segment, the situation that has also observed in previous investigations (6, 13).We also succeeded to detect some behavior similar to mating between the same and different worms although mature eggs did not find. Our finding may be confirming positive potential for cross-fertilization as it was confirm with detection early stages of the development of a shelled egg in the uterus cavity of a monozoic/ vesicular worm cultured in vitro (20).

According to the movie number 1(Movie 1), we speculate some cultivated parasites show tendency to "cross-mating" in culture which could be due to lack of suitable conditions for self-fertilization. Especially the large number of the parasite in culture media may be an advantage in which the small worms can find others. Another our finding is confirmed this theory that insemination between different proglottids of the same strobila is highly unlikely to occur (9). According to movie number 2(Movie 2), we believe the worms have tendency to unsuccessful mating between their individual proglottids. Kumaratilake et al, have believed the type (2) of self-fertilization is unsuccessful because no two proglottids of the same strobila are ever at the same stage of maturation (9). In addition to Kumaratilake's opinion, we are believed that short stature and low number of the segment are important reasons of failure in connection and then mating/fertilization between two ~~strobila~~ segments of an individual worm.

Conclusion

We have observed some possible evidences of unsuccessful mating/fertilization in reared worms. Extremely complexity of requirements for fertilization may cause this physiologic function cannot dissolve production of fertile egg. Regarding the above observations, these parasites show tendency to unsuccessful self-mating/fertilization (type 2) which failure could be due to anatomical position and physiological maturation. Also lack of suitable conditions for self-fertilization causes the worms try to do unsuccessful cross-mating/fertilization in

culture media. More study is necessary to improve our knowledge in this manner.

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