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Cultivation of *Echinococcus granulosus* Larval Stages in Modified Hydatid Cyst Fluid (MHCF)

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Abstract: A fatal zoonosis brought on by the dog tapeworm is echinococcosis (CE). a kind of *Echinococcus*. An efficient therapeutic medication is urgently required because the disease is tough to treat. Cystic echinococcosis has been successfully treated without surgery using chemotherapy, cyst puncture, and PAIR (percutaneous aspiration, injection of chemicals, and reaspiration). Surgery, however, continues to be the most efficient method of cyst removal and can result in a full recovery. In this study, we created *in vitro* simulations of the larval *Echinococcus granulosus* culture media. With the addition of human serum (HS) of blood group AB+, sodium bicarbonate (Na₂SO₄), and glucose, the protoscoleces (PSC) of *E. granulosus* were successfully cultivated in a modified hydatid cyst fluid (MHCF) of *E. granulosus*. For periods ranging from 3 days to 58 days, the larvae were grown in the medium at temperatures between 4 °C and 24 °C. According to the findings, the medium was successful in keeping the larvae alive for as long as possible, and 4 °C was the ideal temperature for them to grow. The ability to create medium for cultivation of parasites that cause human illness is immensely beneficial for researchers because it expands their options for containment and/or eradication strategies and informs them more about the growth and natural history of the parasite.

Keywords: *Echinococcus granulosus*, Parasite, Tapeworm, Zoonosis, Hydatid, Protoscoleces

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Introduction

Cystic echinococcosis (CE) is among the most lethal helminthic diseases that can affect humans (Tappe *et al.*, 2009). The illness is brought on by the dog-tapeworm *Echinococcus granulosus*, which is widespread in the northern hemisphere

(Brunetti *et al.*, 2010; Paoletti *et al.*, 2018). According to current epidemiological study, CE is incredibly common in Central Asia (Dumitru *et al.*, 2015a; Velasco-Tirado *et al.*, 2018). A patient with CE may go up to 15 years without noticing they

have the chronic parasite disease (Mortezaei *et al.*, 2019). CT scanning is 98% accurate and sensitive enough to show daughter cysts. It is the best test for separating hydatid cysts from amebic and pyogenic cysts in the liver is this one (Karabulut *et al.*, 2014).

A CE patient typically seeks medical attention at a late stage when they feel unwell. In this advanced stage, the parasite damages the liver by producing an infiltrating structure made up of multiple tiny vesicles embedded in connective tissue stroma, which makes surgical removal challenging and frequently results in secondary re-infection (Zhang *et al.*, 2015). Ordinarily, surgical removal, if necessary, is the sole curative option for treating AE, and patients must take albendazole (ABZ) and/or mebendazole (MBZ) for at least 2 years following surgical therapy (Loos *et al.*, 2017). Patients with inoperable AE must receive lengthy chemotherapy with ABZ and/or MBZ, typically for the rest of their lives (Mendez *et al.*, 1996; Küster. *et al.*, 2015). Patients with good immune status responded better to albendazole treatment than patients with comorbidities such as diabetes and chronic hepatitis was a negative predictive factor (Dolay and Akbulut, 2014; Kulali *et al.*, 2019). The model we employed in the present study is Low-cost model which is helpful for expediting biological and physiological research as well as the development of anti-CE drugs.

Materials and Methods

At the Fallujah Teaching Medical City General Hospital, human HCF was obtained following the surgical excision of fertilizing cysts from cystic hydatid disease patients. A total of 62 cysts of various sizes were isolated in a germ-free environment. 500 ml of cyst fluid was then taken from these cysts using sterile 20–50 cc needles under sterile conditions. In order to precipitate the larvae, all cyst fluids were centrifuged at 4000 rpm at 4°C for 5 min. Then, the sediments containing the larvae were isolated in 5 ml of liquid at 4°C for 2 days to be used in subsequent

experiments. As for the liquid filtrate it was kept in the freezer under -80 °C until use.

Under a microscope, the viability of the protoscoleces of each fertile cyst was evaluated. Uncertain outcomes were further evaluated after staining with eosin solution (0.1% aqueous), protoscolec-containing hydatid cyst fluid, and letting the mixture settle for 15 min on a microscopic glass slide. The protoscoleces were categorized as viable when they did not pick up the stain and as dead when they did. The modified medium was used to develop the larvae with a viability of 93-95% only.

The modified culture media (diphasic medium) is divided into two phases: (i) the liquid phase which contains a solution of 600 ml of HCF, 400 ml of human serum (AB+ve), 0.5 mg of Na₂SO₄, and 1 g of glucose; and (ii) To make the solid phase, 1.5 g of agarose was dissolved in 100 ml of distilled water, sterilized by boiling, and then cooled at room temperature 45°C. Phosphate PBS solution was then added to the volume in a volume: volume ratio, and AB+ve serum was added and thoroughly mixed after that. Each of the five 50 ml containers underwent progressive incubation at 4°C, 16°C, and 24°C. The percentage of larval vitality was examined over time periods from 3 days to 58 days at each temperature

The medium was reactivated every three days by taking 5 ml of its top layer and replacing it with an equivalent volume of pure or active medium. Each culture was monitored weekly under an optical microscope to check the growth status of the larval vesicles.

Statistical Analysis:

In order to evaluate the data using SPSS 16 with a significance threshold of less than 0.05, descriptive statistics and the Chi-square test were used.

Results and Discussion

E. granulosus metacestodes have been successfully grown *in vitro* in a number of prior experiments (Nabavi *et al.*, 2014). The host support or feeder

cells in the co-culture technique allow the parasite to be kept alive in medium for the several months necessary to develop PSC (Vatankhah *et al.*, 2015). The metacestodes can only be sustained for a short period of time without the feeder cells (Dumitru *et al.*, 2015b; Alghofaily *et al.*, 2017). The results showed that our MHCF modified medium was suitable to keep the larvae alive for as long as possible under different temperatures at range of time from 3-52 days. Our modified medium is an enriched medium which consists of hydatid cyst fluid and addition of human serum (Al Kitani *et al.*, 2015). Human serum contains materials and proteins that allow it to be used in the preparation of media for bacteria (Symeonidis *et al.*, 2013; Jawad *et al.*, 2018) in addition to the fact that the components of the hydatid cyst fluid are comparable to those of human serum (Bulakçı *et al.*, 2016). The physiological relevance of *in vitro* research can be improved by using a growth medium that more accurately reflects the physiological amounts of nutrients, and lately such media types as Plasmax (Liu *et al.*, 2014) and human plasma-like medium (HPLM) were produced (Founta *et al.*, 2016). In addition it consists elements like Ca^{+2} , Na^{+} and K^{+} , GGT (gamma glutamyl transfers (Chauchet *et al.*, 2014). The results of research and studies on the components and biochemical elements present in the hydatid cyst fluid of samples isolated from different hosts (humans, sheep and camels) showed that the fluid contains urea, uric acid, and cholesterol, Albumin, creatin, ALT, AST (Porot *et al.*, 2014). It is the basis of the idea of enriching the liquid medium of the cyst by using human serum. As a result of this function, our MHCF as enriched media contains high nutrition concentration to support the growth (Brumpton *et al.*, 2019). Many previously identified components of HCF proteins are composed of 44% albumin, 39% α -globulin and β -globulin, and 17% γ -globulin. It has been determined that liver HCF and lung HCF from sheep and yak contained 17 amino acids, but the total protein level was very low, equivalent to a level of approximately 1-2% in serum (Frei *et al.*, 2014). The results showed that the percentage of

larvae vitality for all temperatures 4°C, 16 °C, and 24 °C, percentile was high on the third day (93.28%), 90.74%, (80.72%) and the best at 4°C (Table 1).

The findings demonstrated that at all temperatures of 4°C, 16 °C, and 24 °C, had lowest percentage of larval vitality occurred on 58 days (47.7%), (33.44%), and (13.56%) but the best temperature for cultivated the protocol was the 4°C (Table 1).

Organic and inorganic chemicals play an important role in physiological and biological processes, such as the crucial immunity of larvae and cysts (Frei *et al.*, 2014). The present study effectively created enriched culture medium at all temperatures for lengthy periods of time up to 58 days. The growth of the species and its symbiosis in the new media can occur a form of adaptation in the medium due to the metabolic changes in the parasite (Meinel *et al.*, 2018), despite the fact that there are variations between the cyst fluid within the host and in the culture medium (Sharafi *et al.*, 2017; Kamali *et al.*, 2018; Fabbri *et al.*, 2018). The solid phase could be used as a support and nourishment layer by mixing serum with the agarose (Kamali *et al.*, 2018; Fabbri *et al.*, 2018). The lowest vitality percentages for the larvae were in the period of 58 days at all temperatures, but the best was at 4°C (Fig. 1).

Three proteins, β -hemoglobin, albumin, and serum transferrin were found in the cyst fluid (Paoletti *et al.*, 2018; Wen *et al.*, 2019). Transferrins are iron-binding blood-plasma glycoproteins that control the level of free iron in biological fluids and participate in the body's resistance to infection (Garg *et al.*, 2016; Haleem *et al.*, 2018). Thus, the transferrin in cyst fluid is likely able to transport the iron required for the growth of *E. granulosus*. Albumin is the most abundant protein in plasma. Its main function in mammals is to maintain oncotic pressure (Diaz *et al.*, 2015; Liu *et al.*, 2020). It is also a transport protein for fatty acids, unconjugated bilirubin, and thyroid hormones (Hemphill *et al.*, 2014; Cheng *et al.*, 2020). Albumin is also a nutrient for cells

Table 1: Percentage of larvae vitality for all temperatures 4 °C, 16 °C, and 24 °C, percentile was high on the third day (93.28%), 90.74%, (80.72%) and the best at 4 °C. The lowest vitality percentages for those larvae were in the period of 58 days at all temperatures, the best was at 4 °C

Period	N	4 °C		16 °C		24 °C	
		Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
3 days	5	93.28 f	1.347	90.740 e	.829	80.720 f	3.251
7 days	5	87.76 e	2.495	85.940 e	1.814	72.800 e	7.998
14 days	5	81.86 d	2.079	71.420 d	7.926	56.940 d	7.589
28 days	5	72.86 c	4.569	60.120 c	7.814	50.180 c	7.337
48 days	5	62.60 b	3.726	44.460 b	7.256	36.700 b	3.064
58 days	5	47.76 a	3.418	33.540 a	4.350	13.5600 a	2.663
Total	30	74.35 c	16.022	64.370	21.714	51.8167	23.336
Prob.		0.00		0.00		0.00	

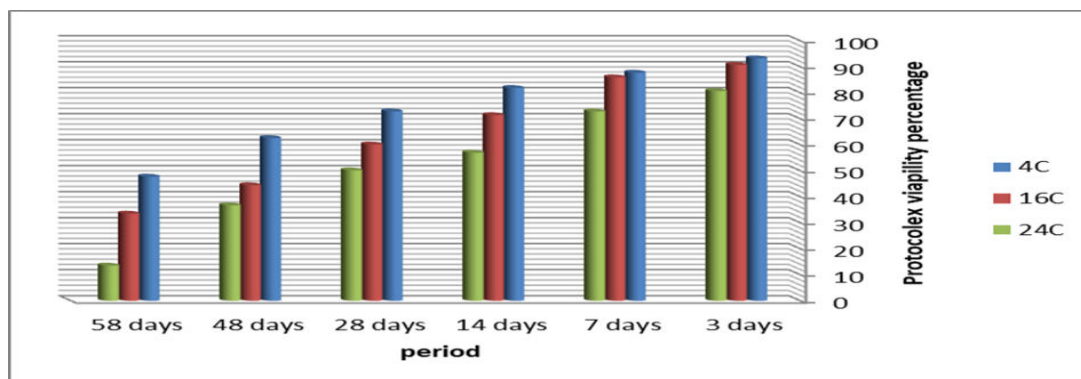


Fig. 1: Changes in viability rate of *E. granulosus* PSC at temperatures of 4°C during the third day (93.28%) had the highest percentage of larval vitality. The lowest vitality percentages for the larvae were In the period of 58 days at all temperatures, but the best was at 4°C.

(Hemphill *et al.*, 2014; Cheng *et al.*, 2020). Human albumin in the cyst fluid provides energy to the larvae (Gottstein *et al.*, 2017). The concentrations of inorganic elements in cyst fluid vary across hosts (Kern *et al.*, 2017; Jansen *et al.*, 2018). The concentration of Na²⁺ in the cyst fluid was approximately half that in healthy human serum, while the levels of K⁺, Mg²⁺, and Ca²⁺ were higher in the cyst fluid (Liu *et al.*, 2020). Other searchers found protoscolex could not be maintained for more than six weeks, indicating that a high concentration of FBS is a key factor for PSC growth (Gao *et al.*, 2021). The disadvantage of other culture methods was the requirement for the continuous presence of host cells (Wang *et al.*, 2016). However, in this study, we produced a simple method which involves a modified medium containing an amount of HFC (60 %) and 40% of human serum which allows PSC to maintain the vitality of the larvae at the highest rate at the best temperature.

Conclusion

An important yet untreated illness is cystic echinococcosis. *In vitro*, we described a simple method in which MHCF promotes healthy growth of *E. granulosus* larval protoscoleces. The biology of the parasite can be studied using *Echinococcus granulosus* *in vitro* and *in vivo* models, an understanding of the larval environment will aid in identifying the essential components of parasite growth and, potentially, in developing novel methods for preventing *E. granulosus* infection.

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