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Development of an *in vitro* culture method for harvesting the free-living infective larvae of *Strongyloides venezuelensis*

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Abstract: An *in vitro* culture technique was established for harvesting *Strongyloides venezuelensis* free-living infective larvae using a nutrient broth medium as a substitute for rat-feces in polyvinyl culture bags (10 × 12 cm). The egg hatch rate (Y) in sterile saline at different incubation temperatures (X) was expressed as the quadratic function, $Y = -0.192X^2 + 8.673X - 19.550$ ($r = 0.901$). The highest (100%) egghatch rate was observed at 25°C. A significant difference ($p<0.05$) in development rate (Y) of free-living infective larvae was observed between different concentrations of nutrient broth (X) which was highest (20.6%) in 0.12% nutrient broth concentrations, incubated at 20°C for 5 days [$Y = -864.032X^2 + 245.995X - 0.560$ ($r = 0.875$)]. Yields (Y) of infective larvae were observed relatively high when the culture medium was incubated at higher temperatures (X) which peaked at 25°C (20.0%) than at lower temperatures, 15°C (10.9%) and 20°C (18.1%) [$Y = -0.189X^2 + 8.387X - 72.795$ ($r = 0.981$)]. The period (Y) required for the development of infective larvae decreased with higher incubation temperatures (X) [$Y = 0.035X^2 - 2.025X + 32.375$ ($r = 0.995$)]. The highest yield (19.2%) of infective larvae was obtained from culture bag inoculated with 15,000 eggs than with below and over 15,000 eggs in 0.12% nutrient broth and incubated at 25°C for 4 days. The newly adapted culture method (from egg to third-stage larva) may be useful as a bio-bar/bioassay system for screening new chemical products, anthelmintics and pesticides, as well as for parasito-immunological studies with *Strongyloides* species.

Key words: *Strongyloides venezuelensis*, free-living infective larvae (L_3), *in vitro* culture, polyvinyl culture bag, bio-bar/bioassay system.

INTRODUCTION

Strongyloides venezuelensis (Brumpt, 1934) (Nematoda: Strongyloididae), a natural intestinal parasite of rats, has been reported from different parts of the world (Brumpt, 1934; Little, 1961; Wertheim and Lengy, 1964; Araujo, 1967; Hasegawa *et al.*, 1988). Morphological and biological characteristics of this nematode species were studied by Little (1966), Hasegawa *et al.* (1988) and Taira *et al.* (1994). Several *in vitro* culture methods for harvesting the free-living infective larvae of *S. venezuelensis* using infected-rat feces have been described elsewhere (Little, 1966; Sato and Toma, 1990; Taira *et al.*, 1994). Such culture methods are: (i) hampered by difficulties in avoiding fecal debris in the larval suspension, (ii) inconvenient to monitor larval development, and (iii) time consuming for recovering infective larvae from the culture

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systems.

Therefore, the present study was conducted to develop a simple and efficient culture technique for harvesting free-living infective larvae of *S. venezuelensis*. Initially, we prepared polyvinyl culture bags with a thin layer of cotton placed at the bottom. The culture bag was inoculated with infected-rat feces, suspended in distilled water and then incubated for the development of infective larvae. However, this method was inconvenient and time consuming for recovering and monitoring the larval development due to the dark color of the culture medium. Subsequently, we inoculated eggs instead of infected-rat feces in polyvinyl culture bags containing a scanty of freshly passed uninfected-rat feces to maintain fecal debris at a minimum level for easy monitoring of larval development. It was, however, still difficult to avoid fecal debris even after the larvae were passaged several times through cotton-wool. This prompted us to use a nutrient broth medium as an alternative to rat-feces not only to avoid the fecal debris, but also to provide a suitable growth environment for *S. venezuelensis* infective larvae.

MATERIALS AND METHODS

Animal

Eight-week-old inbred Sprague-Dawley (SD) male rats, obtained from the Korean Chemical Research Institute, were maintained in our laboratory and used in this study. Animal cages and accessory appliances were cleaned daily to prevent accidental infection through contact with infected feces.

Parasite

S. venezuelensis were provided by the Nippon Veterinary and Animal Science University, Tokyo, Japan, and maintained in our laboratory by serial passage in SD rats. This strain was originally isolated from a wild rat (*Rattus norvegicus*) in Kagoshima Prefecture, Japan, and established as a laboratory strain (Taira *et al.*, 1994).

Cultivation of free-living infective larvae using fecal culture method

Free-living infective larvae (L_3) were initially obtained by fecal culture in polyvinyl bag (10 × 12 cm) as outlined by Prof. H. Saeki. Briefly, a piece of cotton was soaked in infected-rat feces (1.0 g feces/culture bag) suspended in 20 ml distilled water, then placed in a polyvinyl bag (Fig. 1) and incubated at 27°C for 2-3 days. However, this method was labor intensive as a result of excessive fecal materials associated with the larvae when trying to recover the L_3 which developed in the culture bag. Therefore, we used eggs isolated from infected-rat feces by the floatation method using a saturated salt solution. Approximately 10,000 eggs were inoculated in culture bag containing 10 ml sterile saline solution and 0.2 g freshly passed uninfected-rat feces (Fig. 2). Because it was still difficult to isolate the L_3 from fecal debris, we used nutrient broth as an alternative to rat-feces to reduce the turbidity of the rearing medium.

Collection of parasite eggs

The procedure for collecting eggs is briefly described: Ten grams of infected-rat feces were soaked in 160 ml tap water to make a homogeneous suspension and then sieved through a metal net (100-mesh/inch). The suspension was then centrifuged in 50 ml Falcon conical tubes (Becton Dickinson Company, New Jersey, USA) for 7 min at 1,000 *g*. The supernatant was discarded and a saturated salt solution was added to the suspension, then mixed well by vigorous shaking, and centrifuged again for 7 min at 600 *g*. The eggs floated up and were collected by a suction pump. The eggs were then washed four times with a sterile saline solution (0.85%) by centrifugation (single wash at 600 *g* followed by three-washes at 250 *g*) and finally suspended in a known volume of saline solution (0.85%).

Egg hatch rate

The hatch rate of the isolated eggs was observed at different temperatures (0°C, 4°C, 10°C, 20°C, 25°C, 30°C and 37°C) to obtain the maximum number of L_3 from the culture

medium as well as determine the effect of the saturated salt solution (used to collect eggs) on hatch rate.

Cultivation of L_3 in nutrient broth medium

Nutrient broth (Difco, USA) was prepared as a 20% (w/v) stock and autoclaved. Serial concentrations of nutrient broth was then prepared from the stock solution (0.50%, 0.25%, 0.12%, 0.06% and 0.03%) in distilled water. Polyvinyl bags of 10 × 12 cm were prepared and a thin layer of gauze (8-folds; 5 × 10 cm) inserted into each polyvinyl culture bag. Each polyvinyl culture bag was inoculated with 5 ml of nutrient broth and 5 ml of sterile saline suspended with approximately 15,000 eggs and then carefully sealed (Fig. 3). About 10 polyvinyl culture bags were prepared for each concentration of nutrient broth, including the respective controls (by which the nutrient broth was replaced by sterile saline). The polyvinyl culture bags were then incubated at 20°C for larval development to the L_3 stage. During the incubation period, the culture bags were monitored daily for larval growth and development to the L_3 stage. After development of the L_3 in the culture bags, the L_3 were recovered by Baermann's method (Baermann, 1917). This was done at room temperature by allowing one hour for larval migration from the culture materials and for sedimentation. The sediment was then collected and centrifuged at 600 *g* for 7 min. The L_3 recovered from each culture bag were counted under a dissecting microscope. The concentration of nutrient broth found to yield maximum numbers of L_3 were used subsequently with the same number of eggs and incubated at different incubation temperatures (30°C, 25°C, 20°C and 15°C) to determine the effect of temperature on growth and development to the L_3 stage. To obtain a maximum yield of L_3 from a single polyvinyl culture bag, different doses of eggs viz. 2,000, 5,000, 10,000, 15,000, 20,000, 40,000 and 80,000 were inoculated into individual culture bags containing 0.12% nutrient broth and incubated at 25°C.

Comparison between nutrient broth culture and filter paper methods

Larval development to the L_3 stage in nutrient broth culture was compared to the filter paper method described by Sato and Toma (1990) to determine the effectivity of the nutrient broth medium. The filter paper method is briefly described: about 5.0 g of infected-rat feces (35,000 eggs per gram of feces) were collected, soaked in water and then smeared on the upper two-thirds of a large filter paper (15 × 30 cm). The filter paper was then rolled up to stand in an appropriate container containing a small amount of water and incubated at 28°C for 3-4 days. After incubation, the L_3 which migrated to the water pool were collected and counted.

Statistics

Data obtained from this study were analysed by Student t-test and graphs were prepared using polynomial equations (2nd order), CA-cricket Graph III.

RESULTS

Improvement of culture bags

The initial development of polyvinyl culture bags with infected-rat feces was appeared as inconvenient due to the dark coloration of the culture medium (Fig. 1). When isolated eggs were used in the culture bags with a scanty of uninfected-rat feces (Fig. 2), the culture medium became more clean but still found to be unsuitable for use. Use of nutrient broth as an alternative to rat feces resulted in very clean culture medium and larval suspension (Fig. 3).

Hatch rate of parasite eggs

The hatch rate of *S. venezuelensis* eggs in sterile saline at different incubation temperatures provided the quadratic function $Y = -0.192X^2 + 8.673X - 19.550$ ($r = 0.901$), where Y denotes egg hatch rate and X denotes temperature (Fig. 4). Although 25% of the eggs hatched at 37°C, the first-stage larvae died immediately while most of the remaining eggs disintegrated within 48 hr of incubation. Eggs did not hatch in sterile saline at 0°C and

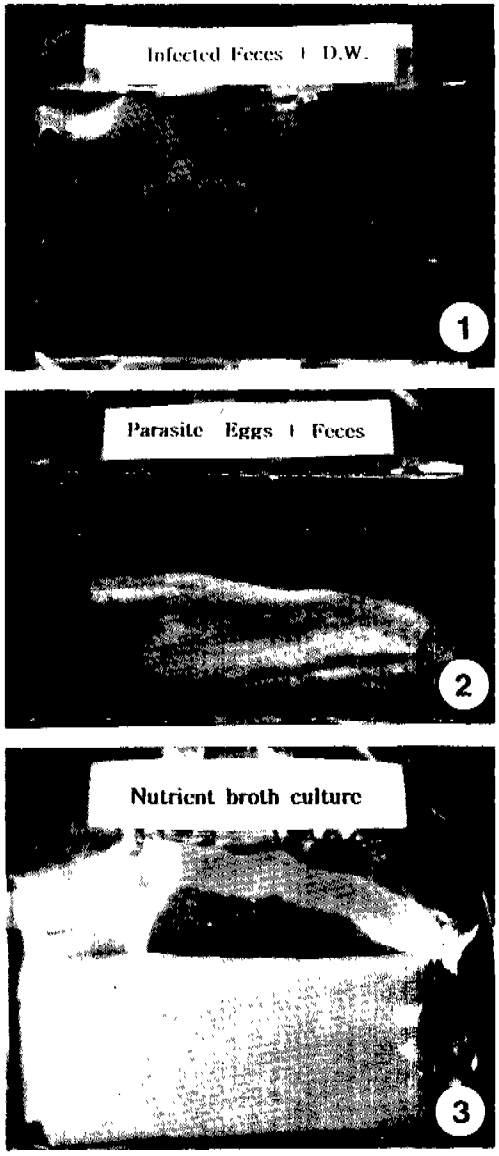

Fig. 1. Polyvinyl culture bags (10 × 12 cm) developed initially for the cultivation of *Strongyloides venezuelensis* free-living L_3 containing infected-rat feces and distilled water, showing dark coloration of the culture medium.
Fig. 2. Polyvinyl culture bag containing parasite eggs and a scanty of freshly passed uninfected-rat feces dissolved in sterile saline. Culture medium is relatively more clean.
Fig. 3. Polyvinyl culture bag containing parasite eggs in nutrient broth showing very clean culture medium.

4°C within 48 hr of incubation. *S. venezuelensis* egg measured 53 ± 3.50 × 26 ± 3.15 μm (Fig. 5).

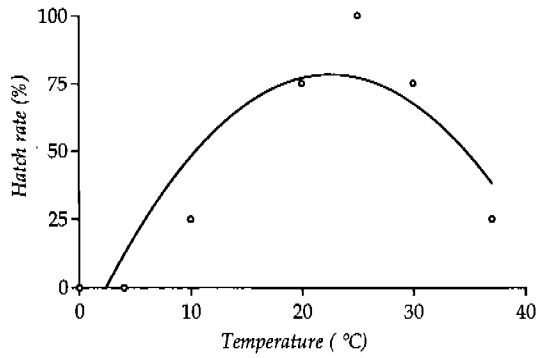

Fig. 4. Hatch rate of *S. venezuelensis* eggs in sterile-saline incubated at different temperatures for 48 hr. Hatch rate of eggs (Y) in relation to incubation temperature (X) is expressed by the following quadratic function. $Y = -0.192X^2 + 8.673X - 19.550$ ($r = 0.901$).

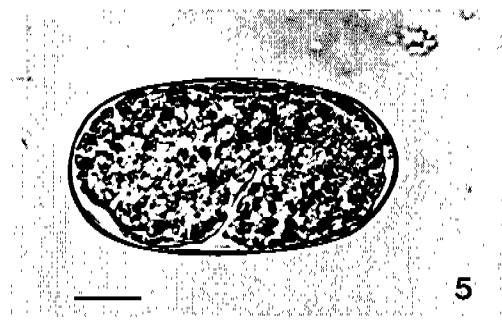

Fig. 5. An egg of *S. venezuelensis* collected from freshly passed rat feces. Bar = 10 μm.

Cultivation of L_3 in nutrient broth medium

S. venezuelensis larvae were grown in nutrient broth culture medium concentrations of 0.06%, 0.12% and 0.25%, and developed to L_3 at the rate of 12.0%, 20.6%, and 12.9%, respectively, within 5 days when incubated at 20°C [$Y = -864.032X^2 + 245.995X - 0.560$ ($r = 0.875$)], where Y denotes larval development rate and X denotes concentrations of nutrient broth (Fig. 6). The larval development rate in different concentrations of nutrient broth were significantly different ($p<0.05$). A relatively higher rate of larval development to the L_3 stage was obtained at 25°C (20.0%) than at 20°C (18.1%), 15°C (10.9%) and 30°C

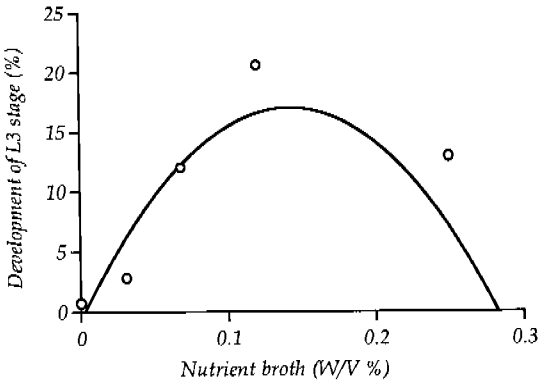

Fig. 6. Development of *S. venezuelensis* L_3 in different concentrations of nutrient broth medium in polyvinyl culture bags and incubated at 20°C. Development rate (Y) of the L_3 according to different concentrations of nutrient broth (X) is expressed by the following quadratic function. $Y = -864.032X^2 + 245.995X - 0.560$ ($r = 0.875$).

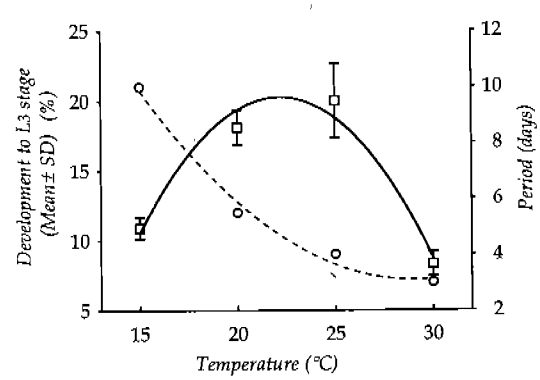

Fig 7. Development rate and period requirement for the *S. venezuelensis* L_3 stage in 0.12% nutrient broth medium incubated at different temperatures. The relationship between larval development rate (Y) (—□—□—) to the L_3 stage and incubation temperatures (X) as well as period (Y) (—○—○—) required for the L_3 formation is expressed by the following quadratic function: $Y = -0.189X^2 + 8.387X - 72.795$ ($r = 0.981$) for development rate and $Y = 0.035X^2 - 2.025X + 32.375$ ($r = 0.995$) for period, respectively.

(8.3%) [$Y = -0.189X^2 + 8.387X - 72.795$ ($r = 0.981$)], where Y denotes larval development rate and X denotes temperature (Fig. 7). The period required for larval development to the L_3 stage was shortened greatly by higher incubation temperatures [$Y = 0.035X^2 -$

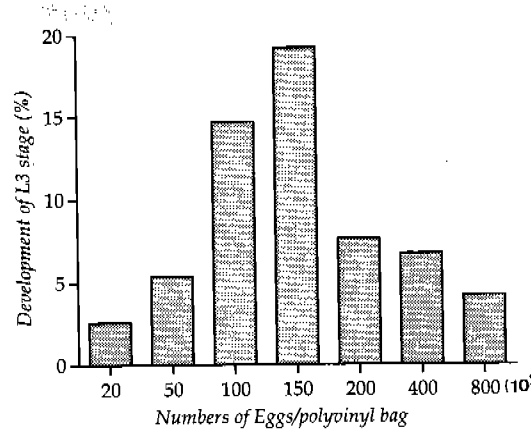

Fig. 8. Yield of the *S. venezuelensis* L_3 according to the number of eggs inoculated in polyvinyl culture bag and incubated at 25°C for 4 days.

$2.025X + 32.375$ ($r = 0.995$)], where Y denotes period and X denotes temperature (Fig. 7). The polyvinyl culture bag inoculated with variable number of parasite eggs were observed to yield maximum L_3 with 15,000 eggs. The yield of the L_3 decreased significantly ($p<0.05$) when more than 15,000 eggs were used in the culture bag (Fig. 8). Mean measurements of *S. venezuelensis* free-living L_3 were: body length, 503.29 ± 31.03 μm; esophagus length, 234.63 ± 15.13 μm; tail length, 52.83 ± 3.12 μm and body width, 15.29 ± 1.34 μm (Fig. 9). The free-living adult females, mean measurements were: body length, 920.62 ± 60.71 μm; esophagus length, 140.14 ± 11.94 μm; tail length, 116.05 ± 10.31 μm; and body width, 50.34 ± 5.45 μm, respectively (Fig. 10), although occasionally developed in the nutrient broth culture, no free-living males were detected in this investigation. Parasitic females measured 2,732.5 ± 169.80, 622.93 ± 49.22, 50.87 ± 3.07 and 41.85 ± 1.83 μm for mean body length, esophagus and tail length, and body width at vulva, respectively (Fig. 11).

Comparison between nutrient broth and filter paper methods

The larval yield in nutrient broth culture method (20.0%) was found about 4-fold higher than the larval yield in filter paper method (5.5%).

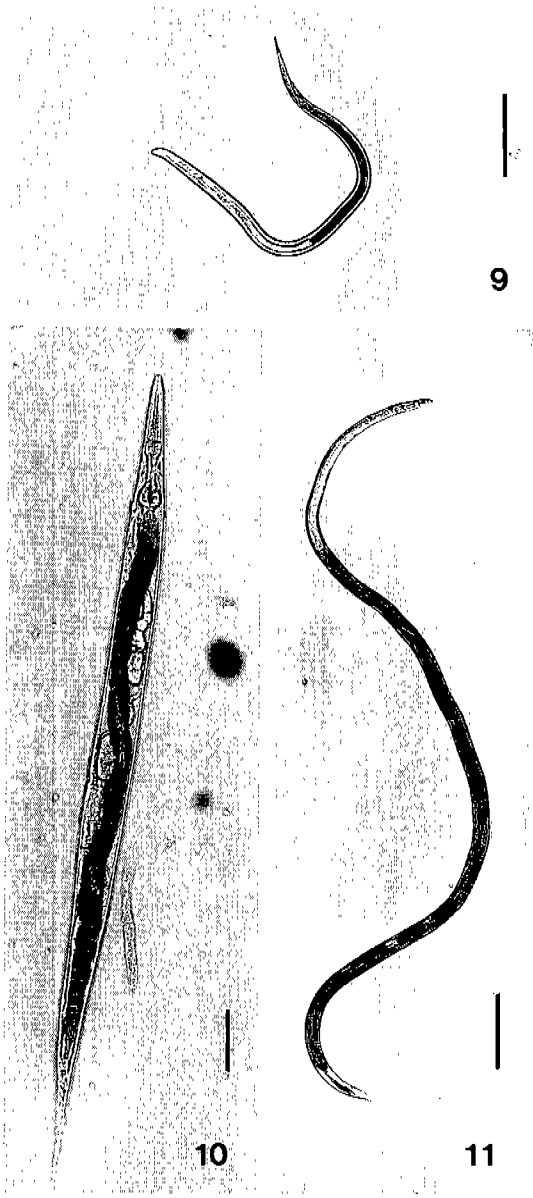

Fig. 9. A free-living L_3 of *S. venezuelensis* harvested from nutrient broth culture. Bar = 100 μm.

Fig. 10. A free-living adult female of *S. venezuelensis* harvested from nutrient broth culture. Bar = 75 μm.

Fig. 11. A parasitic female of *S. venezuelensis* recovered from the small intestine of rat. Bar = 200 μm.

DISCUSSION

S. venezuelensis possesses two different life-cycles, free-living and parasitic. The parasitic females are parthenogenic, living in the mucosal epithelium of the small intestine of their rodent hosts and shedding eggs which are passed in the feces at early cleavage stages of development (Little, 1966). The life-cycle initiates when the L_3 (filariform larvae) penetrate the skin of the host animals. The L_3 migrate to the lungs and subsequently to the small intestine through the tracheal route where they moult and become fourth-stage and sexually mature adult worms. The prepatent period ranges from 6-7 days in rats (Taira *et al.*, 1994).

In the chemical/pharmaceutical industries, the development of new anthelmintics and/or pesticides require a series of screening tests as a preliminary step in their development using a bio-bar/bioassay system to select those drugs/pesticides with the greatest potential. Thus, the development and maintenance of an *in vitro* culture system for free-living infective and/or adult/parasitic stages can provide a bio-bar/bioassay system of great economical importance for chemical/pharmaceutical companies (Douvres and Urban, 1987; Tsuji and Fujisaki, 1994).

The current *in vitro* culture methods described for harvesting *S. venezuelensis* L_3 have several disadvantages. First, the fecal debris cannot be removed easily from the larval suspension since infected-rat feces are used directly in the culture device. This makes the larval suspension opaque, preventing direct observation of the larvae and interfering with the screening tests for potential new chemical products. Second, the status of larval growth and development towards infective stage cannot be visually monitored. Third, the infective larvae must be recovered at specific times to avoid increased larval mortality. Therefore, the aim of the present study was to develop a simple and convenient culture technique that avoids the above mentioned difficulties. In our laboratory, we have successfully developed a nutrient broth culture method that can be used as more

economic and efficient alternative to previously described conventional methods.

A significantly ($p<0.05$) higher rate of larval development to the L_3 stage was observed in 0.25%, 0.12% and 0.06% nutrient broth medium incubated at 20°C for 5 days ($r = 0.875$). The exact reason for such differences in the larval development to the L_3 stage at different concentrations of nutrient broth medium is not known. This is reported for the first time that a chemically-defined medium can be used for harvesting *S. venezuelensis* L_3 from eggs isolated from the infected-rat feces. Although, Tsuji and Fujisaki (1994) used Dulbecco's modified Eagle's medium to harvest the parasitic stage of *S. venezuelensis* from free-living L_3 obtained from fecal cultures by a temperature shift, they evaluated larval development to the parasitic stage in a chemically-defined medium by morphological, protein and antigenicity criteria. However, they did not describe culture medium methods for harvesting L_3 from parasite eggs.

It has generally been established that environmental factors (e.g., temperature and humidity) are important for the development and/or reproduction of parasites. Therefore, the culture bags containing an optimum concentration of nutrient broth (0.12%) were incubated at selected temperatures to determine the effect of culture temperature on the development of the L_3. The temperature ($r = 0.981$) observed for optimum development (20.0%) was 25°C. It was further observed that higher temperatures greatly increased larval metamorphosis to the L_3 stage as evident by higher r-values ($r = 0.995$). These findings are supported by Tsuji and Fujisaki (1994), who reported that the development of the free-living L_3 of *S. venezuelensis* to the parasitic stage was triggered by a temperature shift (from 25°C to 37°C). Present observations further indicate that a change (shift) in temperature might help trigger the biological and/or biochemical mechanisms towards the transformation of the L_3 stage in the life-history of *S. venezuelensis*.

The polyvinyl culture bags (10 × 12 cm) inoculated with approximately 15,000 eggs yielded the highest percent of L_3 while the reason for such variation is not clear, it is thought to be due to intrinsic factors (e.g., concentration of nutrient broth and number of parasite eggs) and extrinsic factors (e.g., availability of oxygen and/or other gases inside the culture bags). The status of the currently established culture technique in terms of larval yield was observed to be about 4-fold higher when compared to the filter paper method.

Present findings suggest that a chemically-defined medium may be useful for harvesting *S. venezuelensis* L_3 from eggstage. However, we did not determine the development of free-living worms to the parasitic stage is possible in this medium as demonstrated by Tsuji and Fujisaki (1994) using a chemically-defined medium with a temperature shift. Although we observed the occasional development of free-living adults, especially females, in the nutrient broth culture medium, we did not observe any free-living male parasites. These findings are in agreement with Hasegawa *et al.* (1988) who reported that free-living males seldom developed in culture medium.

The development of nutrient broth culture method for the cultivation of *S. venezuelensis* L_3 resulted in a very clean larval suspension. The present culture method could be useful as bio-bar/bioassay system for screening a wide variety of chemicals, including pesticides and anthelmintics, facilitate the maintenance *in vitro* of *S. venezuelensis* free-living L_3 in polyvinyl culture bags, and parasito-immunological studies with *Strongyloides* species.

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=초록=

베네수엘라분선충 (*Strongyloides venezuelensis* Brumpt, 1934) 자유생활형 유충의 시험관 내 배양 기술 개발

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Strongyloides venezuelensis 자유생활형 유충을 얻고자, 쥐의 분변 물질을 첨가한 영양 배지를 polyvinyl bag에 넣어 배양하는 배양기술을 확립하였다. 포화식염수법으로 회수한 충란의 부화율은 배양 온도에 따라서 다르게 관찰되었는데 ($Y = -0.192X^2 + 8.673X - 19.550$, $r = 0.901$) 25°C에서 100% 부화율을 나타내었지만 온도가 낮거나 높아도 감소되는 양상을 나타내었다. 회수한 충란은 20°C에서 5일간 관찰하였을 때 자유생활형 유충으로의 발육률은 영양 배지의 농도를 증가시키면 증가하여 0.12%에서 최고치를 보이다가 다시 낮아지는 양상을 보여 ($Y = -864.032X^2 + 245.995X - 0.560$, $r = 0.875$), 감염형 3기 유충(L_3)으로의 발육률은 배양 온도에 따라서 다르게 관찰되었다. 즉, 25°C에서 가장 높아서 20%에 달하며, 15°C는 10.9%, 그리고 20°C에서는 18.1%로 나타났다 ($Y = -0.189X^2 + 8.387X - 72.795$, $r = 0.981$). 그렇지만 감염 유충으로 성장하는데 필요한 기간도 역시 온도의 변화와 관련이 있어 온도가 높으면 높을수록 소요되는 시간이 짧게 나타났다 ($Y = 0.035X^2 - 2.025X + 32.375$, $r = 0.995$). 이상의 결과로 미루어 보아 감염 유충의 최대 회수율 (19.2%)은 15,000개를 polyvinyl bag에 0.12% 영양 배지와 더불어 25°C에서 5일간 배양하였을 때 얻을 수 있었다. 이처럼 새로이 고안한 *Strongyloides* 속의 기생충 배양 기술은 화학 물질, 구충제, 제초제에 대한 선별검사와 기생충과 관련된 면역학적 연구에 생물학적 도구로서 사용될 수 있을 것으로 기대된다. 저자는 *S. venezuelensis*의 국문 이름을 베네수엘라분선충으로 제안한다.

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