# NEMATODE CULTURE WITH SPECIAL REFERENCE TO *ASCARIDIA LINEATA* (SCHNEIDER)

by

ARLIE TODD

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# INTRODUCTION

In a study of the resistance of chickens to parasitism, which is being made at this laboratory by Dr. J. E. Ackert and his associates, the raising of the young nematodes Ascaridia lineata (Schneider) in artificial culture media was deemed desirable. McCoy (1929) raised the free-living larval stage of the dog hookworm, Ancylostoma caninum (Ercolani), on agar cultures of various bacteria and in saline suspensions of living bacteria. Glaser (1931) cultivated the nematode Neoaplectana glaseri Steiner, an oxyurid parasite of the Japanese beetle, (Popillia japonica Newman), a cold-blooded host. Glaser used standard meat infusion agar plates containing 1 per cent dextrose and having a pH value of 7.4. The worms were placed on the surface of the plates together with a water suspension of an actively growing yeast and the cultures kept at room temperature. Glaser was successful in obtaining the entire life cycle of this ovoviviparous worm and later (1932) found it possible to culture the worms indefinitely by adding small amounts of powdered ovarian substances to the culture plates.

The fowl nematode (Ascaridia lineata), however, has no free-living larval stage and, moreover, it does not thrive in a cold-blooded host. Literature upon the culture of such a nematode as A. lineata which hatches from the embryonated egg in the host chicken is lacking. It, therefore, appears that if the young A. lineata are grown outside the body of the host, it will be necessary to develop a special technique. This is the purpose of the present study.

## LIFE CYCLE OF THE NEMATODE

The adult A. lineata are found in the small intestine of chickens, the eggs passing to the outside in the droppings. Given suitable conditions of moisture and temperature, the fertile eggs develop to the infective stage in about 16 days (Ackert, 1931). When given to chickens the embryonated eggs may hatch in either the proventriculus or the duodenum, but the young larvae tend to accumulate in the duodenum a few centimetres behind the entrance of the bile ducts. The first 9 days after hatching, the larvae live in the lumen of the duodenum and in the intervillar spaces. On about the 10th day, the young worms begin to move deeply between the villi and to penetrate the intestinal mucosa destroying Lieberkühn's glands and causing haemorrhages (Ackert, 1923). By the 18th day, the larvae have withdrawn from the mucosa and are to be found free in the lumen of the duodenum where they may develop into adults.

The present study has been concerned chiefly with larvae 18 or more days of age.

## MATERIALS AND METHODS

As the larvae desired for the resistance studies were those of active growing stages, it was planned to infect chicks with embryonated eggs of the nematode and later remove the larvae from the chicks and transfer them to the culture media.

The infective eggs of the A. lineata were obtained by using the culture method of Ackert (1937). Uteri containing fertile eggs were

removed from adult females, placed in Petri dishes and covered with sterile distilled water to which 4 or 5 drops of 2 per cent formalin were added to prevent bacterial or fungous growth. The cultures were incubated approximately 3 weeks at 27-30°C.

The chickens, which were secured as 1 day old chicks, were raised in a screened animal house under conditions modified slightly from those of Herrick, Ackert and Danheim (1923). The following mixed ration was hopper fed: Yellow corn meal, 40 gm.; ground oats, 17 gm.; powdered milk, 6.4 gm.; cracked wheat, 15 gm.; and cod-liver oil, 1.69 gm. In parasitizing the chickens, 50-100 infective eggs were transferred from the culture onto a piece of filter paper which was inserted into the oesophagus of the chicken. To secure larvae, an infested chicken was killed and the small intestine, from the gizzard to the yolk sac diverticulum, was stripped of its mesenteries and removed. This portion was then carefully slit along the side opposite to the mesenteric fold to avoid injuring the worms. As the intestine was opened, the larvae were dropped into a Petri dish containing warm sterile saline solution and incubated at 106-108°F., the body temperature of the chicken. The larvae were left in this solution for about an hour to wash off any clinging ingesta from the chicken intestine. The worms were handled as little as possible to avoid injury and the removal of a mucous-like substance, much like that found on fish, which doubtless has a protective function. The worms were never grasped with forceps, but were washed into the saline solution or transferred with the aid of a curved needle.

In some of the experiments, larvae of several ages were used. The newly hatched larvae in the lumen of the intestine and those partially embedded in the mucosal lining were difficult to recover, being too small and too transparent to be readily observed. It was found that by incubating the slit intestine and its contents in a saline solution at 106-108 F., the smaller larvae would leave the lining of the intestine and could be observed without difficulty with the aid of a binocular dissecting microscope. This appears to be a new method of recovering parasitic larvae partially embedded in host tissue.

In measuring the larvae, the shadows of the worms magnified six times were thrown on the ground glass of a photographic bellows. These shadows were traced for length and measured by a milled wheel so calibrated as to reduce the readings to millimeters. This procedure lessened considerably the experimental error in making the measurements of the larvae.

## TESTS TO FIND A SUITABLE AGAR AND COVER SOLUTION FOR THE NEMATODE CULTURES

The first attempt to culture the larvae of the nematode A. lineata was made with plain agar slants contained in test tubes. Three 20 day old worms were secured from an infested chicken and washed for half an hour in a sterile saline solution. They were then inserted into the tubes and placed in an incubator at a temperature of 106°F. One worm lived for a single day; but the other two were found dead on the second

day. Later tests showed that adult worms died in 15 minutes when left uncovered in an incubator at 106°F. From these results, shown in table 1, it was evident that the worms could not survive without a cover solution.

In another attempt to culture the larvae, eleven 29 day larvae were secured and placed on plain agar plates in 2 inch stender dishes and covered with a sterile isotonic saline solution. The solution was changed twice daily to prevent the growth of fungi and air molds on the cultures. The worms remained moist and active for 4 days (table 1).

It was next determined to test the longevity of worms in isotonic saline solution and in distilled water. Accordingly, eight 34 day larvae were secured from an infested chicken. Four worms were placed in saline solution in a 2 inch stender dish and 4 in distilled water in a similar container. The saline solution and distilled water were changed twice daily. The worms in the saline solution lived 4 days while those in the distilled water lived but 2 days. In later experiments it was found that worms covered with sterile saline solution consistently lived longer than did those kept in distilled water. The longest any worm lived in isotonic saline solution was 7 1/3 days (table 1).

In seeking a satisfactory agar, a test was run on the comparative longevity of worms placed on plain agar and on dextrose cornmeal agar. Eight 52 day larvae, secured from an infested chicken, were given the customary washing, placed on cultures of these two agars and covered with sterile saline solution. The worms on the plain agar remained active for 2 to 4 days while those on the dextrose cornmeal agar in this test remained active 5 days. Later an adult worm was secured which

lived 13½ days on dextrose cornmeal agar when the sterile saline was used as a cover solution. Further experiments showed that worms would live 12 or 13 days (table 1) on dextrose cornmeal agar plates covered with the saline solution. The pH of these cultures was found to be 6.93, unless the cultures were modified by the addition of various foods. The receptacles used were 2 inch stender dishes and the regular Petri dishes.

That a suitable agar and cover solution had now been found is evident from the fact that the A. lineata larvae not only remained moist in the cultures but lived for various periods up to 13½ days. Hence, in all the following experiments the dextrose cornmeal agar plates covered with sterile saline solution were used in all of the cultures.

## EFFORTS TO FIND SUITABLE NUTRIENT MEDIA FOR THE LARVAE

When a suitable agar and cover medium for the cultures had been found, experiments were begun to find nutrient media that would sustain the larvae for some time. The first test was made with a nutrient peptone-infusion broth. Four 42 day larvae were recovered from an infested chicken and, after the customary washing in sterile saline, were placed on plain agar plates and given the peptone infusion broth as a possible food. The worms remained active for 5 days but as they showed no difference in behavior from worms kept in saline solution, they, apparently, had not utilized the broth as food.

Table 1. Showing culture media and nematode larva survival.

| No. of larvae | Age when placed in culture (days) | Agar plate used | Cover solution used | Age of larvae when dead (days) | Days larvae lived in culture |
|---|---|---|---|---|---|
| | 20 | Plain agar | | 21 | 1 |
| 3 | 20 | " " | | 22 | 2 |
| | 20 | " " | | 22 | 2 |
| | 29 | Plain agar | Sterile saline | 33 | 4 |
| 3 | 29 | " " | " " | 33 | 4 |
| | 29 | " " | " " | 33 | 4 |
| | 29 | Plain agar | Sterile saline | 33 | 4 |
| 3 | 29 | " " | " " | 33 | 4 |
| | 29 | " " | " " | 33 | 4 |
| | 29 | Plain agar | Sterile saline | 33 | 4 |
| 3 | 29 | " " | " " | 33 | 4 |
| | 29 | " " | " " | 33 | 4 |
| 2 | 29 | Plain agar | Sterile saline | 33 | 4 |
| | 29 | " " | " " | 33 | 4 |
| | 34 | | Distilled water | 36 | 2 |
| 4 | 34 | | " " | 36 | 2 |
| | 34 | | " " | 36 | 2 |
| | 34 | | " " | 36 | 2 |
| | 34 | | Sterile saline | 38 | 4 |
| 4 | 34 | | " " | 38 | 4 |
| | 34 | | " " | 38 | 4 |
| 1 | 27 | | Sterile saline | 34 | 7+ |
| 2 | 52 | Plain agar | Sterile saline | 54 | 2+ |
| | 52 | " " | " " | 54 | 2+ |
| 2 | 52 | Plain agar | Sterile saline | 56 | 4 |
| | 52 | " " | " " | 56 | 4 |
| 2 | 52 | Dextrose cornmeal | Sterile saline | 56 | 4 |
| | 52 | " " | " " | 56 | 4 |
| 2 | 52 | Dextrose cornmeal | Sterile saline | 57 | 5 |
| | 52 | " " | " " | 57 | 5 |
| | 70 | Dextrose cornmeal | Sterile saline | 80 | 10 |
| 3 | 70 | " " | " " | 82 | 12 |
| | 70 | " " | " " | 82 | 12 |
| 2 | 70 | Dextrose cornmeal | Sterile saline | 78 | 8 |
| | 70 | " " | " " | 82 | 12 |
| 2 | 82 | Dextrose cornmeal | Sterile saline | 90 | 8 |
| | 82 | " " | " " | 90 | 8 |
| *2 | 82 | Dextrose cornmeal | Sterile saline | 94 | 12+ |
| | 82 | " " | " " | 90 | 8 |
| *1 | 94 | Dextrose cornmeal | Sterile saline | $108\frac{1}{2}$ | $13\frac{1}{2}$ |

* Adult nematodes.

Later, a group of 69 day old larvae was placed on dextrose corn-meal agar and covered with the peptone-infusion broth. As controls, another group of larvae of the same age and source was placed on the same type of agar plate and covered with sterile saline solution. In this experiment, the worms in the broth lived for 8 days, while those in the sterile saline solution lived 12 days (290 hours). In both cases, the cover solutions were changed twice daily. From these results it is evident that the larvae were not utilizing the broth as a food.

In a second experiment of this series, thirty-four 41 day old larvae were placed on agar plates with mucus scraped from the intestine of their host. Sterile saline solution was used as a cover medium. The worms lived 4½ days (110 hours) in the culture, but gave no evidence that they had utilized the mucus as a food.

In another effort to find a suitable food for the larvae, it was decided to use bacterial suspensions in an isotonic saline solution. The suspension of Escherchia coli, a bacterium found in the intestine of chickens, was stained with eosin and methylene blue so that the suspension could be easily observed if taken into the digestive tracts of the worms. In the first test, 4 worms placed in the suspension lived 3 days when the culture was maintained at 106-108°F. In a second test, 12 worms placed in similar suspensions lived 4½ days. Although the bacteria were swallowed, as shown by the colored digestive tracts, the worms gave no evidences of growth.

It was observed that so long as the worms were alive their cuticle did not stain.

In another test, eight 97 day worms were secured from an infested chicken, washed as usual, placed in a sterile Petri dish and covered with a broth made from the regular chicken feed (described elsewhere). The broth was an extract made by boiling the feed in distilled water and filtering and sterilizing the liquid material after the solid particles were removed. Once every day the broth was changed and the worms removed to sterile saline during the night to prevent the growth of fungi or bacteria in the cultures. These worms lived 7 days (163 hours) but gave no evidence of growth. This test was repeated several times and in each instance approximately the same results were obtained.

The results of these tests are in accord with those of Li (1933) who upon feeding charcoal and starch granules to chickens and finding both in the worms' intestines concluded that A. lineata fed essentially on the intestinal contents of the host, though he thought that damage to the intestinal wall was possible. The results also confirm those of Ackert and Whitlock (1935) and Ackert and Freeman (1936) who found that, when chickens infested with A. lineata were removed from feed and kept alive by water per os and by intramuscular glucose injections, the worms did not grow. Furthermore, Ackert (1938) carried out a series of tests to ascertain if A. lineata might be a tissue or lymph feeder. He found that when worms were placed in the body cavities of chickens they did not grow although they were in the presence of both tissue and lymph. As Li states, the worms appear to feed essentially on the intestinal contents of their host, but it seemed from the writer's tests with broth made from the regular chicken feed that the worms must be in the presence

of food which is ready to be assimilated.

These efforts to find nutrient media for the worms showed that although the larvae could live up to 8 days in the broth made from the chicken feed and up to 7 days in the peptone infusion broth, they lived only $4\frac{1}{2}$ days in the bacterial suspensions and in the mucus from the chicken intestine. At the same time worms lived for 12 days when they were simply covered with the saline solution and placed on agar plates. It thus appeared that the nutrient medium to be used would be a broth or a solution containing food material ready to be assimilated.

## EXPERIMENTS TO INDUCE GROWTH OF THE LARVAE IN CULTURES

After techniques for agar plates and cover solutions were developed and nutrient solutions had been prepared that would maintain the A. linesta larvae for many days, the next step was to ascertain if the larvae would grow in the nutrient solutions. Accordingly, the larvae were measured before being placed in the cultures and immediately upon their removal from them.

When the worms were measured it was found that during the 24 hour period after the larvae were transferred from the chicken intestine to the culture they lost 1 or 2 mm. of length. This may have been due to shock, or possibly the worms were undergoing a "lag-phase" comparable to that found in bacteriology when bacteria are transferred to fresh cultures. Some of the worms would soon recover and start growing while others failed to do so.

## Larvae Cultured in Hens' Eggs

The first evidences of growth were obtained from worms cultured in an incubating hen egg. Two 19 day larvae, measuring 22.9 and 24.07 mm., respectively, were placed inside an egg which was immediately sealed with albumin and eggshell. One day later the egg was opened and it was found that the larger worm had grown .99 mm. The smaller worm, on the other hand, had lost .4 mm. The following day the smaller worm died having lost an additional .43 mm. However, the larger worm survived 4 days and, when it was found dead, measured 29.95 mm., a gain of 5.88 mm. in 4 days or a rate of growth of approximately $1\frac{1}{4}$ mm. per day. Ackert (1931) found that larvae of this age (19 days) in their natural habitat grew at the rate of 1.5 mm. per day.

The experiment was repeated with two other larvae which made growths of 2.6 mm. and 1.8 mm., respectively, in 2 days. Although the worms made definite growth no further efforts were made to culture A. lineata larvae in hens' eggs because of the difficulties involved in observing the worms.

## Larvae Cultured in Carbohydrate Solutions

It was next determined to try combinations of carbohydrates as food for the worms. One food was composed of 20 gm. of starch, .5 gm. of maltose, and .25 gm. of peptone in 50 cc. of distilled $H_2O$. This solution had a pH value of 6.2 which was more acid than the 6.7 average of

the worms' habitat in the chicken (Ackert, 1931). No worms lived longer than 2 days in this solution and no evidences of growth were found.

Later 20 gm. of starch and 10 gm. of dextrose were mixed in 50 cc. of distilled water. This solution had a pH value of 6.23. Larvae placed in this medium, on dextrose cornmeal agar plates and incubated at 106-108°F. gave evidences of growth. In table 2, which gives the results of these tests, it may be seen that a larva 20 days of age measuring 20.4 mm. grew 3.8 mm. in length in a period of 2 days. This was an increase of 18.6 per cent. A group of 3 larvae 23 days of age and from 21 to 28½ mm. in length were placed in this culture. After 3 or 4 days, the worms had increased in length from 4.7 to 5.9 mm. or from 20.7 to 22.4 per cent. Of two other groups of this size and age, one increased in length while the other group lost.

During the remainder of the experiment, 10 larvae 26 days of age were placed in the cultures. These lived from 2 to 7 days and gave mixed results. Of the 10, 4 gained in length, 4 lost size, and 2 neither gained nor lost in size.

As controls for the 24 worms cultured in this experiment, 4 larvae from corresponding hosts were placed in isotonic saline solution for from 2 to 5 days. In all cases, the larvae not only failed to grow but actually became shorter.

The results of this experiment in which larvae from 20 to 26 days of age were placed in a culture of starch-dextrose solution on dextrose cornmeal agar plates show that many of the larvae in the nutrient solutions gained in length to the extent of from .4 to 22.4 per cent,

Table 2. Showing data on the growth of Ascaridia lineata larvae in a starch-dextrose solution on dextrose-cornmeal agar plates.

| Number of Ascaridia larvae | Age of larvae when placed in cultures (days) | Length of larvae when placed in cultures (mm.) | Time of larvae in cultures (days) | Length of larvae when removed from cultures (mm.) | Gain in length (mm.) | Loss in length (mm.) | Per cent gain in length |
|---|---|---|---|---|---|---|---|
| 2 | 21 | 12 | 2 | 9.7 | 0.0 | 2.3 | |
| | | 15 | | 10.7 | 0.0 | 4.3 | |
| 1 | 20 | 16.3 | 1 | 15.5 | 0.0 | 0.8 | |
| 1 | 20 | 21.8 | 1 | 22.1 | 0.3 | 0.0 | 1.4 |
| 1 | 20 | 20.7 | 2 | 20.5 | 0.0 | 0.2 | |
| 1 | 20 | 21.3 | 4 | 17.0 | 0.0 | 4.3 | |
| 1 | 20 | 20.4 | 2 | 24.2 | 3.8 | 0.0 | 18.6 |
| | | 21.0 | 3 | 25.7 | 4.7 | 0.0 | 22.4 |
| 3 | 23 | 22.7 | 3 | 27.5 | 4.8 | 0.0 | 21.1 |
| | | 28.5 | 4 | 34.4 | 5.9 | 0.0 | 20.7 |
| | | 19.6 | 4 | 21.5 | 1.9 | 0.0 | 9.7 |
| 3 | 23 | 26.9 | 4 | 27.1 | 0.1 | 0.0 | 0.4 |
| | | 29.2 | 4 | 32.3 | 3.1 | 0.0 | 10.6 |
| | | 26.1 | 5 | 25.4 | 0.0 | 0.7 | |
| 3 | 23 | 27.8 | 4 | 26.5 | 0.0 | 1.3 | |
| | | 28.0 | 6 | 26.3 | 0.0 | 1.7 | |
| | | 26.6 | 6 | 22.2 | 0.0 | 4.4 | |
| 3 | 26 | 32.0 | 6 | 32.0 | 0.0 | 0.0 | |
| | | 32.2 | 6 | 35.9 | 3.7 | 0.0 | 11.5 |
| | | 25.7 | 6 | 25.8 | 0.1 | 0.0 | 0.4 |
| 3 | 26 | 31.8 | 6 | 33.4 | 1.6 | 0.0 | 5.0 |
| | | 36.4 | 6 | 36.4 | 0.0 | 0.0 | |
| 2 | 26 | 25.3 | 7 | 22.8 | 0.0 | 2.5 | |
| | | 37.0 | 7 | 42.7 | 5.7 | 0.0 | 15.4 |
| 2 | 26 | 29.6 | 2 | 23.6 | 0.0 | 6.0 | |
| | | 30.7 | 5 | 26.4 | 0.0 | 4.3 | |
| | Control larvae in isotonic saline solution. | | | | | | |
| 1 | 25 | 35.4 | 2 | 33.5 | 0.0 | 1.9 | |
| | | 31.5 | 4 | 31.3 | 0.0 | 0.2 | |
| 3 | 29 | 41.0 | 5 | 40.7 | 0.0 | 0.3 | |
| | | 53.2 | 5 | 45.7 | 0.0 | 7.5 | |

whereas, the control worms not only did not gain but lost in length. Table 2 shows, likewise, that a number of larvae in the nutrient solution also lost size. The cause for this phenomenon has not been determined. All larvae were handled in as nearly the same manner as possible

Larvae Cultured in Isotonic Salt-Dextrose Solutions

In another effort to find a suitable food for the larvae, it was decided to try the isotonic salt-dextrose solution used by Stunkard (1932) in his attempt to culture Crepidobothrium lonnbergi, a cestode from the intestine of the cold-blooded amphibian, Necturus. The solution which had a pH value of 6.4 was composed of the following:

| | |
|---|---|
| Sodium chloride | 2.25 gm. |
| Calcium " | 0.06 gm. |
| Potassium " | 0.10 gm. |
| Sodium bicarbonate | 0.04 gm. |
| Dextrose | 0.62 gm. |
| Distilled $H_2O$ | 1000 cc. |

When first used, the solution was not diluted and none of the worms lived more than a day. It was observed that when the isotonic salt-dextrose was undiluted an adult worm lived but one day in the solution and when the worm was left longer in the solution it swelled and burst. The experiment was then repeated. Worms placed on dextrose cornmeal agar plates and covered with the solution diluted four times gave evidence of growth (table 3). At the same time, other worms of the same age and from the same chicken placed in sterile saline as controls lost length as consistently as the others gained.

As shown in table 3, 24 larvae ranging in age from 23 to 36 days, were placed in isotonic salt-dextrose solution on dextrose cornmeal

agar plates. Of the 24 worms cultured, 20 made growths ranging from .2 mm. to 25.9 mm. in from 2 to 11 days. The two extremes represented were worms of the same age, from the same chicken, and received the same treatment in placing them in the cultures. They differed in size by only 4.8 mm. and the smaller larva made the most growth. Only one worm of the 20 which grew, failed to grow more than 1 mm. Fourteen of the worms which grew made growths of more than 5 mm. and 6 of them grew more than 10 mm. Similarly the worms made percentages of gain ranging from .4 per cent to 53.8 per cent, and of the 20 worms which grew, 11 made gains of over 20 per cent, 7 made increases of over 30 per cent, and one gained 53.8 per cent in length.

Four of the worms lost length in this type of culture, the losses ranging from 1.3 mm. to 9.2 mm. in from 2 to 11 days, even though they appeared to receive the same treatment as the worms which grew and in some cases were in a culture with another worm which grew.

Nine worms used as controls by being placed in the isotonic salt solution did not grow and all lost lengths ranging from .2 mm. to 7.5 mm. in from 2 to 4 days.

Thus the evidence from table 3 shows that the larvae maintained themselves successfully up to 11 days in the isotonic salt dextrose solution and made growths up to 25.9 mm. in length.

Table 3. Showing data on the growth of *Ascaridia lineata* larvae in salt-dextrose on dextrose cornmeal agar plates.

| No. of larvae | Age when placed in cultures (days) | Length of larvae placed in cultures (mm.) | Time of larvae in cultures (days) | Length of larvae when removed from cultures (mm.) | Gain in length (mm.) | Loss in length (mm.) | Per cent gain in length |
|---|---|---|---|---|---|---|---|
| 1 | 23 | 27.0 | 4 | 35.4 | 8.4 | 0.0 | 31.1 |
| 1 | 25 | 29.5 | 3 | 36.0 | 6.5 | 0.0 | 22.3 |
| 1 | 25 | 28.4 | 3 | 37.9 | 9.5 | 0.0 | 33.5 |
| 2 | 25 | 27.1 | 3 | 33.1 | 6.0 | 0.0 | 22.1 |
| | | 27.5 | 8 | 40.4 | 12.9 | 0.0 | 46.9 |
| 2 | 33 | 22.3 | 3 | 24.0 | 1.7 | 0.0 | 7.6 |
| | | 22.5 | 3 | 28.2 | 5.7 | 0.0 | 25.7 |
| 2 | 33 | 33.1 | 6 | 28.0 | 0.0 | 5.1 | 0.0 |
| | | 54.0 | 6 | 44.8 | 0.0 | 9.2 | 0.0 |
| 2 | 36 | 48.6 | 11 | 53.8 | 5.2 | 0.0 | 10.7 |
| | | 51.2 | 10 | 56.7 | 5.5 | 0.0 | 10.7 |
| 2 | 36 | 44.7 | 7 | 51.1 | 6.4 | 0.0 | 14.3 |
| | | 50.4 | 11 | 52.7 | 2.3 | 0.0 | 4.6 |
| 2 | 36 | 50.8 | 4 | 52.0 | 1.2 | 0.0 | 2.4 |
| | | 54.1 | 9 | 73.4 | 19.3 | 0.0 | 35.7 |
| 2 | 36 | 52.2 | 11 | 50.9 | 0.0 | 1.3 | 0.0 |
| | | 52.9 | 11 | 53.1 | 0.2 | 0.0 | 0.4 |
| | | 39.9 | 3 | 51.8 | 11.9 | 0.0 | 29.9 |
| 3 | 36 | 41.5 | 4 | 56.0 | 14.5 | 0.0 | 34.9 |
| | | 48.1 | 9 | 74.0 | 25.9 | 0.0 | 53.8 |
| | | 26.3 | 2 | 28.0 | 1.7 | 0.0 | 6.5 |
| 4 | 36 | 30.5 | 2 | 32.0 | 1.5 | 0.0 | 4.9 |
| | | 37.4 | 2 | 36.0 | 0.0 | 1.4 | 0.0 |
| | | 48.0 | 5 | 66.3 | 18.3 | 0.0 | 38.1 |
| | Control larvae in isotonic saline solution. | | | | | | |
| 1 | 33 | 33.6 | 3 | 33.4 | 0.0 | 0.2 | |
| 2 | 33 | 26.8 | 4 | 24.2 | 0.0 | 1.6 | |
| | | 45.5 | 2 | 38.0 | 0.0 | 7.5 | |
| 2 | 36 | 48.8 | 4 | 48.1 | 0.0 | 0.7 | |
| | | 50.3 | 4 | 50.0 | 0.0 | 0.3 | |
| | | 39.3 | 4 | 38.6 | 0.0 | 0.7 | |
| 3 | 36 | 41.9 | 4 | 39.0 | 0.0 | 2.9 | |
| | | 44.0 | 4 | 39.5 | 0.0 | 4.5 | |
| 1 | 36 | 49.0 | 4 | 48.0 | 0.0 | 1.0 | |

## Larvae Cultured in Animal Protein-Carbohydrate Solutions

As the fowl diet, and presumably that of the worms contains animal protein, the next culture prepared contained liver-extract along with a mixture of starch and dextrose. The mixture contained 20 gm. of starch, 5 gm. of dextrose, 3 gm. of liver extract and had a pH value of 5.35. Worms placed in these cultures did not grow as vigorously as did those in the isotonic salt-dextrose solution; the gains recorded were in most cases rarely above 5 mm. Having obtained growth with this food, the experiment was repeated with a group of worms in sterile saline solution as controls.

In the second test, three 29 day larvae were placed in the food cultures and two larvae of the same age and from the same chicken were placed in the saline solution. The worms on the food grew 5.37, 5.5 and 6.43 mm., respectively, while the controls lost 5.2 and 9.2 mm., respectively, during the 4 day period of the test (table 4).

Altogether 17 worms were placed in this type of culture and of this number 14 made growths of 1.5 mm. to 6.1 mm. in periods of 2 to 5 days. The 14 worms which grew made gains of from 4.1 per cent to 23.6 per cent, as shown in table 4.

Only three worms lost length but these losses ranged from 2.6 mm. to 11.7 mm. in periods of 4 to 6 days.

The results of these tests showed that the larvae would grow in a solution containing animal protein and carbohydrates but not as successfully as in the isotonic salt-dextrose solution.

Table 4. Showing data on the growth of Ascaridia lineata larvae in a starch, dextrose, liver-extract mixture on dextrose cornmeal agar plates.

| No. of larvae | Age when placed in cultures (days) | Length of larvae placed in cultures (mm.) | Time of larvae in cultures (days) | Length of larvae when removed from cultures (mm.) | Gain in length (mm.) | Loss in length (mm.) | Per cent gain in length |
|---|---|---|---|---|---|---|---|
| | | 10.3 | 2 | 11.8 | 1.5 | 0.0 | 14.5 |
| 3 | 28 | 17.4 | 3 | 21.5 | 4.1 | 0.0 | 23.6 |
| | | 24.4 | 5 | 27.0 | 2.6 | 0.0 | 10.7 |
| | | 28.8 | 4 | 34.2 | 5.4 | 0.0 | 18.8 |
| 3 | 29 | 35.9 | 4 | 41.4 | 5.5 | 0.0 | 15.3 |
| | | 37.3 | 4 | 43.7 | 6.4 | 0.0 | 17.2 |
| | | 49.1 | 4 | 37.4 | 0.0 | 11.7 | |
| 3 | 34 | 49.2 | 6 | 46.6 | 0.0 | 2.6 | |
| | | 54.0 | 4 | 48.0 | 0.0 | 6.0 | |
| 2 | 46 | 51.1 | 2 | 57.2 | 6.1 | 0.0 | 11.9 |
| | | 52.1 | 2 | 57.8 | 5.7 | 0.0 | 10.9 |
| | | 50.4 | 2 | 54.4 | 4.0 | 0.0 | 7.5 |
| 3 | 46 | 51.9 | 2 | 55.8 | 3.9 | 0.0 | 7.5 |
| | | 52.2 | 2 | 57.8 | 5.6 | 0.0 | 10.7 |
| | | 48.8 | 3 | 52.8 | 4.0 | 0.0 | 8.2 |
| 3 | 46 | 50.0 | 3 | 55.4 | 5.4 | 0.0 | 10.8 |
| | | 65.3 | 3 | 68.0 | 2.7 | 0.0 | 4.1 |
| | Control larvae in isotonic saline solution. | | | | | | |
| 2 | 29 | 44.4 | 4 | 39.2 | 0.0 | 5.2 | |
| | | 44.0 | 4 | 34.8 | 0.0 | 9.2 | |

## DISCUSSION

Mention has been made of a mucous-like covering of the worms (A. lineata) which may have a protective function. In the early experiments the worms were removed from the slit fowl intestine by grasping them gently with a pair of forceps or by picking them up with the fingers. When the worms were changed to fresh cultures they were also handled in this manner. Frequently the cultures were contaminated by fungi and molds which doubtless were introduced during the inspections necessary to study the larvae. Later the worms were found with patches of molds which appeared to be growing on the parts of the worms that were touched in handling. These growths, it seemed, were a possible cause of their deaths. As a result of these observations, methods were adopted which facilitated the handling of the worms, and, at the same time, preserved this mucous-like envelope. After these methods were adopted no more worms were attacked by fungous growths.

## SUMMARY

1. In connection with studies on the resistance of chickens to parasitism, efforts were made to raise young Ascaridia lineata (Schneider) in artificial cultures. During the experiments more than 800 larvae were secured from infested chickens and cultured in vitro.

2. Evidence that the worms swallowed substances in the culture media was available from their colored digestive tracts after the immersion of the larvae in a stained suspension of bacteria. The body walls of living larvae did not stain.

3. The most effective basic culture medium consisted of dextrose cornmeal agar plates covered with sterile saline solution. Petri and stender dishes were used as culture dishes.

4. To the basic medium various nutrient solutions were added. Growth of the nematode larvae was secured by the addition of each of three different combinations of foods: (a) dextrose-starch, (b)dextrose-starch-liver extract, and (c) an isotonic salt-dextrose solution.

5. With the addition of the dextrose-starch solution to the basic medium, larvae made growths ranging from 0.29 to 5.7 mm. or growths of 0.4 to 22.4 per cent. When the starch-dextrose-liver extract solution was added to the basic medium, larvae increased in length from 1.5 to 6.43 mm., or 4.1 to 23.6 per cent. The isotonic salt-dextrose solution added to the basic medium enabled the larvae to make growths ranging from 0.4 to 25.9 mm. or increases in size of 0.4 to 53.8 per cent.

6. Evidence of growth was also secured when larvae were cultured in incubating hens' eggs.

7. Worms lived in the cultures from 2 to $13\frac{1}{2}$ days.

8. It was found that the larvae possessed a mucous-like covering which served a protective function. Absence of patches of this material led in many cases to the death of the worms due to growths of molds or fungi.

9. A new method was developed for recovering parasitic larvae which were partially embedded in host tissue. The tissue, in this case the slit intestine of the chicken, was covered with sterile saline solution and incubated at 106-108°F. for half an hour or so when the larvae withdrew their anterior ends from the mucosa. The larvae, which are small and rather transparent, could be readily observed by the use of a binocular dissecting microscope.

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