FACTORS AFFECTING *IN VITRO* CULTIVATION OF *HISTOMONAS MELEAGRIDIS* ISOLATED FROM EGGS OF *HETERAKIS GALLINARUM* AND HISTOMONOSIS IN TURKEYS

by

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INTRODUCTION

The importance of the eggs of Heterakis gallinarum in the transmission of Histomonas meleagridis is well documented. With the exception of the report by Ruff, et al (1970), no work has been done on the isolation and culturing of Histomonas from the heterakid egg.

In vitro cultivation of Histomonas provides for studying its biochemical requirements and the role of cecal bacteria for its cultivation, survival in culture and pathogenicity. The current study investigated (1) possible routine systems for isolating histomonads from the heterakid egg, (2) some cultural requirements for best in vitro growth, (3) the role of cultural techniques on subsequent pathogenicity of the organisms, and (4) comparative morphological studies of the isolated histomonad with Histomonas meleagridis from stock culture.

LITERATURE REVIEW

The earliest published reference to enterohepatitis (histomonosis) was that of Cushman (1893) who noted diseased livers in turkeys. He did not determine if the condition was caused by disease organisms or if it was due to overfeeding or some other unfavorable circumstance. Smith (1895) described oval bodies (6 to 10 μ in diameter) in sections of diseased livers as a species of ameba, Amoeba meleagridis. Tyzzer (1919) studied its developmental phases and noted its flagellate characteristics. He named it Histomonas meleagridis. There is still controversy on whether the organism is an ameba, a flagellate, or an amebaflagellate (Reid 1967, Schuster 1968). The organism is an ameba throughout its life cycle, but in the cecum of the host it possesses one or more flagella which are lost when the parasite invades the host's tissues. In culture, Histomonas has the morphology of the lumen dwelling forms (Bishop 1938, Levine 1961, Lund, et al 1967).

TRANSMISSION OF HISTOMONAS

The role of H. gallinarum in the transmission of enterohepatitis was first elucidated by Graybill and Smith (1920). They found that the disease could be transmitted by feeding larvated H. gallinarum eggs to turkeys. They believed that the disease resulted when the invading larvae broke down tissue and provided a portal of entry for the protozoa already present in the ceca. Tyzzer, Fabyan and Foote (1921) confirmed the work of Graybill and Smith. They noted that it was not necessary to feed the protozoan with the ova to produce the disease.

The theory that the H. gallinarum egg may harbor H. meleagridis inside its shell also received strong support from Tyzzer and Fabyan (1920) when

they found that H. gallinarum eggs sterilized for 3 days in 1.5% nitric acid were capable of transmitting enterohepatitis when fed to turkeys. Tyzzer (1934) failed to produce the disease in susceptible birds by feeding them non-larvated H. gallinarum eggs or male Heterakis. When Swales (1948) ground up larvated and non-larvated H. gallinarum eggs and fed them to turkey poults, he was not able to obtain an infection, whereas enterohepatitis was produced in turkeys by washed embryonated cecal worm eggs given per os, by intra-cecal inoculation of washed infective larvae, and by the intra-cecal inoculation of larvae that had been sterilized in hydrogen peroxide. It was suggested by Swales (loc cit) that the role of the cecal worm in the transmission or initiation of the disease is dependent on living larvae. Glaser (1921) and Tyzzer (1926) could not find H. meleagridis in H. gallinarum eggs. Niimi (1937) believed that structures 1.0 to 1.4 μ in diameter which he saw in the eggs in the ovary, oviduct and uterus of H. gallinarum were H. meleagridis. Wehr (1954) found development of enterohepatitis and large proportions of deaths when susceptible turkeys were fed feces containing cecal worm eggs deposited on soil 7 months earlier by birds harboring the causative protozoans.

Heterakis gallinarum larvae and adults have been examined in search for an etiological agent of enterohepatitis. Tyzzer (1934) saw protozoa in sections of the epithelium of the gut of 10 and 12-day-old H. papillosa (= H. gallinarum) developing in chickens and in a 21-day-old worm from a turkey. Desowitz (1950) found a multinucleate cell in the gut epithelium of an adult female H. gallinarum. Connell (1950) saw refractive swellings in the cuticle of second-stage H. gallinarum larvae. Blackhead-free and blackhead carrying stocks of H. gallinarum second-stage larvae were established and a comparative study made. Abnormalities were observed in a proportion

of living larvae from blackhead-carrying stocks. Similar abnormalities were not observed in larvae from blackhead-free stocks. The abnormalities consisted of cuticular swellings, diffuse thickenings in intestinal or esophageal regions, and proctodeal and caudal abnormalities. Kendall (1957) records with a photomicrograph and a camera lucida drawing the occurrence of parasites resembling H. meleagridis in young larvae of H. gallinarum.

The most complete search for the protozoan in H. gallinarum was that by Gibbs (1958, 1962), who presented good evidence that he actually saw H. meleagridis in H. gallinarum males and females as well as in eggs of the nematode. Infected male worms contained histomonads in the gut wall and the wall and lumen of the reproductive system. Female worms infected with H. meleagridis showed the organism throughout the reproductive system. Springer (1969) transmitted H. meleagridis to poults by oral inoculation with recently harvested, intact male Heterakis worms. When female worms carrying non-embryonated ova were administered, no histomonosis resulted.

The study by Ruff, McDougald and Hansen (1970) proved that Histomonas could be isolated from the egg of H. gallinarum, thus supporting the contention that the organisms can be transmitted via embryonated eggs. Histomonads were isolated from eggs of H. gallinarum by culturing artificially hatched eggs in a modified DeVolt's alkaline serum medium. The presence of the protozoan was established by microscopic examination and confirmed by producing histomonosis in turkeys by intra-cecal inoculation with quantities of the cultures.

Lund and Burtner (1957) estimated that less than 1 out of every 200 heterakid eggs was positive for Histomonas. Vatne (1963) found 50% of the chicks positive for blackhead when given a dose of 100 eggs. With a dose of 600 eggs, he found 90% chicks positive for histomonosis. Ostlind (1966)

reported an average incidence of 10 to 24.6% for histomonosis among experimental chickens. The 10% incidence may have been due to attenuation of the histomonad by storage of the eggs in the refrigerator for almost a year (7-9 C).

Farmer, Hughes and Whiting (1951) have reviewed and described in detail the signs of the disease. In turkey poults, there is an invasion of the cecal mucosa resulting in necrosis of the tissue. This dead tissue is sloughed and forms a soft 'cheese-like' core in the lumen of the ceca. During the process of invasion, some histomonads penetrate the blood vessels of the ceca and are transported to the liver by the hepatic portal veins where they are trapped by the reticuloendothelial cells of the liver (Clarkson 1961). Further focal necrosis takes place in the liver resulting in round greenish-yellow necrotic lesions with depressed centers. Diarrhea and the presence of yellowish feces precede either death or recovery. Upon necropsy, the presence of inflamed ceca with 'cheesy-cores' and 'typical liver lesions' have been assumed to be diagnostic. Reid (ibid) however, has attacked this diagnostic procedure and has pointed out a number of other common diseases, such as trichomonosis, tuberculosis and mycotic diseases, which have similar signs.

MEDIA

Histomonas meleagridis has been cultured in the presence of bacteria with variable success since 1924. Drbohlav (1924) successfully maintained the organism on slants of coagulated egg white covered with blood bouillon containing 1% peptone. Tyzzer (1934) obtained good growth in a medium composed of buffered egg albumin agar covered with 5% sterile horse serum in saline.

Bishop (ibid) successfully cultured the organism from liver lesions using Dobell and Laidlaw's inspissated horse serum (1:8). DeVolt and Davis (1936) used Locke-egg-serum medium with Locke's solution containing turkey serum or a combination of serum and albumin. DeVolt (1943) obtained luxuriant growth on a medium composed of Locke's solution containing 2% turkey serum and 2% of 0.2N sodium hydroxide. Since unprecipitated serum supported more growth than precipitated serum, sodium hydroxide was added to prevent precipitation of serum proteins during autoclaving. In addition, rice starch was added to the medium before inoculating with histomonads.

Various methods have been suggested for continued maintenance of the organism in culture. Delappe (1953) used penicillin and streptomycin in Laidlaw's medium to facilitate *in vitro* isolation of the organism. Lesser (1960 a,b) cultured the organism without viable bacteria in tissue culture media using Selas filtered cream, antibiotic-treated nutrient broth culture of turkey cecal bacteria and penicillin G potassium, dehydrostreptomycin sulfate and mycostatin. Lesser (1961) also cultured *H. meleagridis in vitro* axenically using a modified culture medium enriched by fresh hamster liver tissue and metallic ions. He also had success with hamster kidney tissue (Lesser 1963). Bradley (1963) successfully grew the organism in avian embryos. Lesser (1964) found that pure cultures of *Escherichia coli*, *E. freundii*, *Proteus mirabilis* and a lactobacillus failed to support *in vitro* growth of *Histomonas*.

Larson (1964) was unsuccessful in culturing *H. meleagridis* in Tyzzer's, Laidlaw's and Delappe's mediums, but was able to grow the organism through 56 transfers for 11 months in DeVolt's medium (without glucose). Ostlind (ibid)

cultured the organism successfully using a modified DeVolt's medium. He reduced the turkey serum to 15 ml (1.5%) and used double distilled water at a pH 9.25-9.50. Ruff (1968) further modified DeVolt's medium by using double glass distilled, deionized water and 10 ml (1.0%) turkey serum at a pH of 9.0-9.25. Ostlind (loc cit) found that 0.5 g of finely ground Bacto rice powder per tube of medium and an undefined amount of ground animal charcoal increased the longevity of cultures. Ruff (loc cit) used lump charcoal to facilitate easier counts and better multiplication of the organism. McKee (1969) found that the amount of starch and charcoal used in the medium affected the multiplication of the organism. A significantly lower growth level was observed in 5 cc of medium containing 3.5 mg of starch (finely ground Bacto rice powder) than in medium containing 7.0 mg or 14.0 mg of starch. When .803 g of animal charcoal was used in the medium, multiplication was less than that in cultures receiving .155 g or .418 g of charcoal.

SYNERGISM

The need for a study of the relationship of bacteria to protozoa in the etiology of histomonosis has been apparent for some time. One of the first steps in an inquiry into the microbiology of blackhead would be to obtain information about the normal flora of healthy turkeys.

Menes and Rochlin (1929) stated that the intestinal flora of domestic birds (chickens, geese, and turkeys) is chiefly E. acidi-lactici and Streptococcus faecalis. Kucel (1934) reported finding 3 species of bacteria in the intestinal tract of 25 healthy turkeys: Bacterium coli, E. acidi-lactici and S. faecalis. No attempt was made to identify anaerobes. Harrison and Hansen (1949) conducted a detailed study in order to determine the bacterial flora of the ceca

of healthy turkeys. The flora of birds of different flocks was found to vary in no greater degree than the flora of birds of the same flock. Members of the following 12 genera have been identified: Bacillus, Bacterium, Borrelia, Corynebacterium, Escherichia, Lactobacillus, Micrococcus, Paracolobactrum, Pediococcus, Sarcina, Spirillum and Streptococcus. More than 20 species have been isolated, however, the great bulk of the bacterial population is composed of but a few genera and species. Anaerobic lactobacilli were the most numerous bacteria in the cecal feces of the turkeys tested, making up about 50% of the total cultivable flora. Gram-positive, nonsporeforming rods other than lactobacilli and anaerobic streptococci are the next in predominance. Coliforms and enterococci exist in relatively small numbers, each usually comprising less than 1% of the flora. It is apparent from this study that anaerobic bacteria are in the majority in the cecal flora and that almost the entire flora is composed of gram-positive organisms.

Harrison, Hansen and DeVolt (1954) identified the bacteria associated in blackhead liver abscesses with H. meleagridis. E. coli was encountered most often, 1 out of every 3 livers. Other bacteria identified include: Pseudomonas aeruginosa, Salmonella newport, Micrococcus flavus, M. pyogenes var. albis and aureus, S. faecalis, S. inulinaceus, S. liquefaciens, Lactobacillus acidophilus, L. bifidus, L. fermenti, Diplococcus magnus, Clostridia perfringens and Borrelia spirochetes.

It appears that the bacteria which may join, at one time or another, histomonads in liver lesions, are not necessarily species most evident in normal cecal flora. This contention is supported by the presence in liver abscesses of such bacteria as M. aureus and P. aeruginosa which were not encountered in preliminary studies of normal cecal flora. Furthermore, although E. coli was the most frequently encountered bacterial species in

liver abscesses, it was found to be one of the less dominant species in normal cecal flora.

Doll and Franker (1963) inoculated bacteria-free and conventional turkeys with bacteriologically sterile heterakid eggs containing H. meleagridis. Only 1 of 12 gnotobiotic turkeys showed any signs of the disease, and none died of the disease. Of 12 conventional turkeys, 11 developed histomonosis and died. These results suggest that either the infectivity of Heterakis or the pathogenicity of Histomonas, or both, may be related to normal host flora. Franker and Doll (1964) studied the effects of some cecal bacteria on pathogenicity of H. meleagridis. No indication of histomonad infection was observed in birds harboring either Bacillus cereus or L. fermenti. A single case of liver infection was noted in 8 birds harboring S. faecalis. Both liver and cecal involvement were seen in 4 of 8 birds harboring E. intermedia, and in 1 animal there were cecal lesions without hepatic infection; but no typical caseous exudates in ceca. With E. intermedia and B. cereus as introduced cecal flora, cecal involvement was enhanced. In hosts harboring E. intermedia and L. fermenti, however, cecal infection was similar in most respects to that noted in their conventional counterparts.

Bradley and Reid (1966) applied Koch's postulates for suspected disease organisms to the combination of H. meleagridis and E. coli and each of the 4 postulates was fulfilled, indicating that this combination of organisms represented a true etiological pattern for infectious histomonosis of turkeys. Negative results for disease production were obtained with H. meleagridis alone; H. meleagridis and Aerobacter cloacae + S. faecalis; or H. meleagridis and Proteus mirabilis, as inocula for gnotobiotic turkeys. Similarly negative results were obtained from the administration of killed or filtered E. coli + viable H. meleagridis.

MATERIALS AND METHODS

Medium:

Histomonads were cultured and maintained in DeVolt's alkaline serum medium (DeVolt, ibid) as modified by Ostlind (ibid) and Ruff (ibid) in order to increase longevity of the organisms and reproducibility of results. DeVolt's medium consists of the following: 1000 ml distilled water, 9.0 g NaCl, 0.2 g CaCl, 0.4 g KCl, 0.2 g $NaHCO_3$, glucose, 20 ml turkey serum, and 20 ml of 0.2N NaOH to adjust to pH 9.0-10.0. Modifications included the use of double glass distilled deionized water, 10 ml of turkey serum and no glucose in order to reduce growth of associated cecal bacteria. The pH was adjusted to 9.0-9.25 using 0.2N NaOH and a pH meter.

A Cornwall automatic pipetter was used to dispense 5 ml of the media into each 16x75 mm Kimax screw cap tube. The culture tubes were then autoclaved at 120 C, 15 lb pressure for 20 minutes. After autoclaving, the tubes were refrigerated until use. Medium over 3 months old was discarded as Larson (ibid) and Ruff (ibid) found such tubes of medium yielded variable results.

Serum Preparation:

Blood was obtained from heavy white turkeys (90 to 120 days old) by slitting the jugular vein and collecting the blood in Petri dishes. The dishes had been coated with physiological saline (0.85%) to prevent blood clots adhering to the sides.

The dishes containing blood were covered and allowed to set undisturbed for several hours before the serum was carefully removed using a Pasteur pipette. The serum was divided into groups, clear and cloudy. Hemolyzed serum was not used as Larson (ibid) indicated that it was detrimental to

multiplication of Histomonas. When the sera was centrifuged, cloudy sera generally cleared. Sera was centrifuged at 75 and 160 g, respectively, for 15 minutes at 0 C. The supernatant was removed and again divided as to clear and cloudy. All tubes were dated and labeled as to quality. Sera were handled carefully to prevent undue contamination prior to autoclaving in the DeVolt's medium. All sera was stored in a freezer (-16 C) until used.

Charcoal:

The exact function of charcoal added to the tubes of medium is not completely understood. It has been suggested that it absorbs harmful products of metabolism because tubes with charcoal support growth longer than those without charcoal. McKee (ibid) suggests that an overabundance of charcoal might inhibit high multiplication rates of histomonads but also tends to sustain a culture over a longer time. Lump animal charcoal yielded less variable multiplication in the tubes and made counts easier than the finely ground charcoal previously used by Larson (ibid) and Ostlind (ibid). Sargent lump charcoal was placed in 150x16 mm Kimax screw cap tubes and sterilized in dry heat at 160 C for 2 hours. The charcoal was stored at room temperature until needed. A normal amount in a culture tube was enough to fill the bottom curvature of the tube.

Starch:

An optimum amount of starch was added to the culture tubes for continuous and consistent multiplication of the organisms in vitro. McKee (ibid) found cultures receiving 3.5 mg of starch multiplied at a significantly slower rate than did the cultures receiving 7.0 mg or 14.0 mg of starch. The cultures receiving 7.0 mg of starch multiplied most rapidly except on day 15 post-inoculation when this culture group was surpassed by the cultures receiving 14.0 mg. Bacto rice powder, finely ground with a mortar and pestle, was

placed in 60x17 mm homeopathic vials and capped with aluminum foil. Vials were sterilized in a dry heat oven at 150 C for 2 hours, care being taken not to char the starch.

Charcoal and 7.0-14.0 mg of starch were added to warmed tubes of media just before the organisms were inoculated. The contents of the tubes were mixed gently by inverting, the organisms added and the tubes incubated at 39.5 C in a water bath.

Glassware:

All glassware, except the Petri dishes, was acid cleaned. Pasteur pipettes used for removal of culture samples and graduated pipettes were placed in an acid bath (H_2SO_4-$K_2Cr_2O_7$). After soaking for 24 hours, they were transferred to an upright automatic pipette washer and washed with tap water for at least 16 hours before they were removed and dried. The Pasteur pipettes were placed in large test tubes which then were plugged with cotton and capped with aluminum foil; all other pipettes were placed in metal canisters. All pipettes were dry heat sterilized for 2 to 3 hours at 170 C.

All screw cap tubes were placed in an acid bath of concentrated HNO_3 for a minimum of 24 hours. They were rinsed 9 times in tap, twice with distilled, and twice with double glass distilled deionized water, placed upside down in racks and allowed to dry. The tubes were sterilized along with the medium when needed. Caps for these tubes were cleaned in the same manner as the tubes, except for the acid procedure.

Counting Protozoa:

A Levy hemacytometer chamber with Neubauer ruling was used for counting histomonads. There is a space 0.1 mm thick under the cover glass when it is in place. The ruled area of the slide has a surface area of 9 sq mm.

To calculate the number of organisms per milliliter, the number of

organisms counted over a 9 sq mm was divided by 9 (the number of organisms in 1 sq mm), then multiplied by 10 (the number of organisms in 1 cu mm since layer is 0.1 mm thick) and then multiplied by 1,000 for the number of one cubic centimeter or one milliliter. This translates into the formula:

$$N/9\ (10)\ (1{,}000) = \frac{N10^4}{9} = \text{organisms/cc}$$

To fill the hemacytometer, a Pasteur pipette, held in a horizontal position to prevent settling of the organisms, was filled with a thoroughly mixed culture suspension, and the tip, after the first few drops of culture fluid was discarded, was touched to the edge of the platform. A thin layer of fluid was allowed to flow under the cover glass. If too much fluid was added, the counting chamber was cleaned and refilled. The chamber was cleaned out with Kimwipes after the population of organisms was enumerated in each culture tube. All counts were recorded as number of organisms per milliliter.

Transfer of Organisms:

Transfers to fresh medium were made every 4-7 days by removing a sample from a culture tube with a sterile Pasteur pipette and placing 2-3 drops into a new tube of complete medium (media containing both charcoal and starch). About 3,000-5,000 Histomonas, per hi-dry microscope field, were transferred to fresh medium when making subcultures.

Host:

The turkeys used in this investigation were of the Broad Breasted White variety (Nicholas strain) and were purchased as 1-day-old poults from a commercial hatchery. All birds were vaccinated intranasally against Newcastle disease, raised in electric brooders, fed antibiotic-free commercial ration, and watered and fed ad libitum up to and during the experimental period.

Anesthetization for Laparotomy:

Birds were anesthetized with anesthesia-grade diethyl ether. The criteria of a good anesthetic are those of individual variability, duration of anesthesia, subsequent livability, and absence of deleterious effects. On the basis of ease, rare fatalities, rapid anesthesia, and rapid post-operative recovery, this technique was employed throughout the course of this study.

To a wide-mouth jar partially packed with cotton was added 10 ml of diethyl ether. The head of the bird was placed in the mouth of the jar without blocking the passage of air into the container. Proper anesthetization had been obtained when the animal's breathing was deep and rhythmic and the legs could be extended and would drop slowly to the rear when released. The head of the bird was then removed from the ether atmosphere. If the breathing became rapid and shallow, the bird was over-anesthetized and was removed from the ether atmosphere to fresh air. If the breathing became exceedingly rapid and muscle tension became noticeable, the bird was regaining consciousness and ether was again administered. Less than 1% mortality due to this type of anesthesia was encountered during this study.

Laparotomy:

An incision approximately an inch long was made with a scissors along a line from the base of the sternum to the ischium of the pelvic bone. The wound was spread by means of clamps and the ceca were located by means of a blunt probe. After examination or injection, the ceca were replaced in the abdominal cavity. The incision was closed with 11 mm wound clips.

Source of Culture:

Histomonas meleagridis were obtained from a stock culture of embryonated eggs of H. gallinarum (1,000 ova/ml) furnished by Salsbury Laboratories.

They cultured the eggs in a 2½% formalin solution. Before shipment they replaced the formalin solution with water. However, some formalin was still present thus maintaining sterility of the culture. The sterility of the culture was verified by broth inoculation. Thus, no bacteria were present in the medium containing embryonated eggs used in this study.

Isolation of H. meleagridis from Embryonated eggs of H. gallinarum:

At the beginning, and intermittently during these experiments, a dose of approximately 200 embryonated H. gallinarum eggs were inoculated per os into young turkey poults in order to verify the pathogenicity of the culture. Eleven to 14 days post-infection, the infected turkeys were sacrificed and H. meleagridis plus associated bacteria were isolated from the ceca. The livers of these birds were necrotic, thus verifying the pathogenicity of the strain.

Experiment 1:

Three sets (A,B,C) of embryonated heterakid eggs of approximately 10,000 (Set A), 20,000 (Set B), and 40,000 (Set C) in number were placed in centrifuge tubes. A 1:1 ratio of 3% sodium hydroxide and 3% sodium hypochlorite (Chlorox) was added to each of these tubes to sterilize and deshell the eggs. Eggs were exposed to the deshelling solution for 24 hours (Hansen, et al 1956). Each set of eggs was washed 4 times by centrifugation with 0.85% physiological saline. After centrifugation, the eggs were concentrated into the following amounts of physiological saline: Set A- 9 ml, Set B- 15 ml and Set C- 12 ml. Each set of eggs was placed in a sterile Ten Broeck tissue grinder and ground until the vitelline membranes were ruptured. Tubes of sterile DeVolt's medium received aliquots (larvae and debris from the eggs) from the Ten Broeck tissue grinder. The size of the aliquots which were taken from the tissue grinder to inoculate tubes of complete medium are given in Table I.

Cecal bacteria are thought to be necessary for the survival and growth of histomonads in DeVolt's medium. A laparotomy was conducted on a young uninfected turkey poult and 5 ml of nutrient broth was injected into the distal end of the right cecum with a 5 ml syringe and 20 gauge needle followed by aspiration of the injected fluid back into the syringe. One ml of the aspirated material was injected into a dilution bottle containing 100 ml of nutrient broth which was then subcultured daily for 2 days while kept incubated at 39.5 C. This source of turkey cecal bacteria was added to the tubes of DeVolt's in varying quantities as given in Table I.

To preclude the possibility of contamination, all steps, beginning from the step where the eggs were placed in the Ten Broeck tissue grinder through the addition of cecal bacteria, were conducted in an isolation chamber equipped with an ultraviolet light source. Before this, and all experiments, the isolation chamber was disinfected with a Roccal solution (400 ppm) and then exposed for 15 minutes to UV light.

All experiments included 1 or 2 sterility checks using thioglycollate broth or millipore filter (MF). The former method was conducted by inoculating 100 ml of thioglycollate broth with a .5 ml aliquot from the Ten Broeck tissue grinder and incubating at 39.5 C; clear broth indicating sterility.

Millipore filters were used in the first 3 experiments in addition to the fluid thioglycollate medium to insure detection of all cecal bacterial contaminations. Millipore filters are porous membranes composed of cellulose esters. When fluids pass through MF;s, all particles, bacteria and cells larger than the pores are retained on the filter surface and lie in a plane where they can be readily examined and counted. Type HA filters (0.45 microns) retain nearly all known non-viral microorganisms. To insure the filtration of the smallest bacteria, the Type GS filter was used, with a pore size

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

TABLE I

Regimen for inoculating DeVolt's medium, Experiment 1.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)*
1	0	0
2	0	2
3	1,100	4
4	1,100	3
5	1,100	2
6	2,200	1
7	2,200	0
8	0	0
9	0	2
10	2,660	4
11	2,660	3
12	3,990	2
13	3,990	1
14	3,990	0
15	0	0
16	0	2
17	6,600	4
18	6,600	3
19	6,600	2
20	6,600	1
21	6,600	0

* Disposable Pasteur pipette used for administering drops

0.22 μ ± .02 microns. A 47 mm diameter MF holder was employed for liquid filtration in conjunction with a vacuum flask. The filter holder was wrapped in aluminum foil and the entire assembly was autoclaved for 15 minutes at 121 C. A slow autoclave exhaust was used to allow the assembly to cool to room temperature before use. A small sample (0.5 ml - 1 ml) from the Ten Broeck tissue grinder was passed through the filter system. The MF, bearing organisms on its surface, was plunged into a liquid growth medium (thioglycollate broth) where detection rather than enumeration is indicated.

Microscopic examination for histomonads in Experiment 1 was made of tubes at 7 days postinoculation, after which time subcultures were made.

Experiment 2:

Seven tubes of DeVolt's medium each received 2,000 ground heterakid eggs/ml of physiological saline. The isolation technique for histomonads and sterility checks was conducted as in Experiment 1, with the exception of the aliquot size from the Ten Broeck and drops of cecal bacteria added to each tube, (Table II).

Microscopic examination for histomonads began at 4 days postinoculation, when subcultures were made. Histomonads isolated by this technique were inoculated via laparotomy into the right ceca of 12-day-old-turkey poults. After inoculation, each bird was isolated in a cage. If any deaths occurred, the day was noted and necropsies were done to determine if characteristic "cheesy cecal cores" and lesions of histomonosis were present.

Experiment 3:

This experiment followed the same regimen as did Experiments 1 and 2 with the following exceptions; 10 tubes of DeVolt's medium were each inoculated with 1,000 ground heterakid eggs, and the drops of bacteria differed as shown in Table III. Microscopic examination for histomonads began at 4

TABLE II

Regimen for inoculating DeVolt's medium, Experiment 2.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)
1	0	2
2	1,000	4
3	1,000	3
4	1,000	2
5	1,000	1
6	2,000	3
7	2,000	2

TABLE III

Regimen for inoculating DeVolt's medium, Experiment 3.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)
1	0	2
2	1,000	2
3	1,000	2
4	1,000	2
5	2,000	2
6	2,000	2
7	2,000	2
8	2,000	2
9	2,000	2
10	2,000	2

days postinoculation, and histomonads isolated by this technique were inoculated via laparotomy into 19-day-old turkey poults.

Experiment 4:

In Experiment 4, 10 tubes of DeVolt's were inoculated with 2,000 ground heterakid eggs, and each tube received 2 drops of cecal bacteria. The only change in this experiment from prior ones was that the Heterakis eggs were not sterilized and deshelled in a 3% sodium hydroxide-hypochlorite solution. The eggs were taken out of refrigeration, washed 4 times by centrifugation with physiological saline and placed in a Ten Broeck tissue grinder. Microscopic examination began at 9 days postinoculation and histomonads isolated were inoculated via laparotomy into 2-month-old turkeys. Beginning with this experiment, the only sterility check was the thioglycollate broth.

Experiments 5 and 6:

Experiments 5 and 6 utilized a fresh culture of turkey cecal bacteria. The bacteria used in the first 4 experiments came from the original isolation and were merely subcultured in nutrient broth every 2 days. The same technique was employed in isolating these bacteria, and 2 days after inoculating into nutrient broth, 2 drops were added (in both Experiment 5 and 6).to 5 tubes of DeVolt's which each received 2,000 ground heterakid eggs.

Experiment 7:

The mode of infection used in this experiment was that of injecting Histomonas cultures into the brachial wing veins of 3-month-old turkeys. Four birds were chosen at random and 3 of them received by injection with a 25 gauge needle and 2 ml syringe, 1 ml of culture containing 35,000 known virulent histomonads. The remaining bird received in the same manner, a 1 ml injection of 1,000 artificially hatched histomonads. After inoculation, each bird was isolated in a cage and closely observed.

Experiment 8:

Experiment consisted of 4 trials. Three factors were common in all 4 trials, they were; 1) each tube received 2 drops of a 48-hour-old cecal bacteria culture, and 2) a 1 ml aliquot from the tissue grinder was used to inoculate thioglycollate broth for the sterility check, and 3) microscopic examination for histomonads began at 4 days postinoculation.

In the first trial, 10,000 heterakid eggs were concentrated into 10 ml of physiological saline and were ground in the tissue grinder. Five tubes of DeVolt's medium received ground debris; one tube received 2.5 ml while the remaining 4 received 1.5 ml.

In trial 4, 10,000 Heterakis eggs were concentrated into 6 cc and ground in the tissue grinder. All 5 tubes received a 1 ml inoculation from the tissue grinder.

Experiment 9:

Experiment 9 followed the same regimen as the other experiments with the exception of the source of cecal bacteria. In this experiment, a small sample of fresh cecal feces was inoculated into warm physiological saline which served as the source of cecal bacteria. Fifteen-thousand eggs were concentrated into 11 ml and 5 tubes of DeVolt's were inoculated with 2 ml aliquots from the Ten Broeck tissue grinder. Microscopic examination for histomonads began at 4 days postinoculation.

Morphological study:

Histomonas was stained with iodine using Tyzzer's method (1934). A small drop of culture was placed on a slide and covered with a cover glass and after a few minutes, when the organisms had extended their pseudopodia, full strength iodine solution (5% potassium iodide in saline solution with iodine added to saturation) was added at the edge of the cover glass. As

the iodide penetrated the material it fixed the protozoa, clearly revealing pseudopodia and flagella. Dry films were fixed in Schaudinn's and stained in Giemsa's stain. While flattening and distortion of structures occurred, these features often worked to advantage in the study of minute, closely placed structures. The cytoplasm showed a wide variation in its reaction to the Giemsa stain (Tyzzer, loc cit).

RESULTS

Experiment 1 was conducted in the attempt to confirm by culture that Histomonas is in the heterakid egg. Microscopic examinations of the sediment from ground embryonated eggs revealed free and crushed larvae, embryonated eggs and ruptured shells. The results after culturing this material are presented in Table IV. Growth of H. meleagridis was detected in 2 of 15 tubes after 1 week incubation. At 25 days incubation, 5 of 15 tubes had histomonads but the populations never exceeded 3,500 organisms/cc. Two of 3 tubes inoculated with sediment from the Ten Broeck but not with cecal bacteria were positive for histomonads, whereas, those inoculated only with bacteria were negative 25 days postinoculation. Tubes receiving 6,000 ground heterakid eggs supported the greatest numbers of histomonads for the longest period of time. Both sterility checks indicated that the techniques were adequate in all experiments except in Experiment 4.

Experiment 2 investigated the isolation and culturing of greater numbers of Histomonas from heterakid eggs as well as identification of the organism by host inoculation. Microscopic examination of the contents of the tubes in Experiment 2 revealed histomonads in 3 of 6 tubes after 4 days incubation (Table V). At 10 days incubation, 4 of 6 tubes had histomonads. Tubes receiving 1,000 ground eggs and 2 drops of cecal bacteria sustained 24,000 Histomonas per cubic centimeter, a concentration higher than that reported in Experiment 1. Further identification of these isolates was done by inoculating various cultures into 8, 12-day-old poults, via laparotomy and direct cecal injection. Four poults received 1 ml of culture contents from a 4-day-old culture containing 20,000 histomonads and of these 4 poults, 1 died a day after surgery. Two poults were inoculated via the same routes with contents from cultures showing no growth and the remaining 2 poults were

TABLE IV

Confirmation by culture that *Histomonas meleagridis* can be isolated and cultured from eggs of *Heterakis gallinarum*, Experiment 1.

Set	Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation				
				7	12	16	25	29
A	1	0	0	0	0	0	0	0
	2	0	2	0	0	0	0	0
	3	1,100	4	0	+	0	0	0
	4	1,100	3	+	++	+	0	0
	5	1,100	2	0	0	0	0	0
	6	2,200	1	++	+	0	0	0
	7	2,200	0	0	0	0	+	0
B	8	0	0	0	0	0	0	0
	9	0	2	0	0	0	0	0
	10	2,660	4	0	0	+	0	0
	11	2,660	3	0	0	0	0	0
	12	3,990	2	0	+	0	0	0
	13	3,990	1	0	+++	+	0	0
	14	3,990	0	0	0	0	+	+
C	15	0	0	0	0	0	0	0
	16	0	2	0	0	0	0	0
	17	6,600	4	0	+	+	+	0
	18	6,600	3	0	+++	++	+	+
	19	6,600	2	0	0	0	++	0
	20	6,600	1	0	0	0	0	0
	21	6,600	0	0	0	0	0	0

0 no growth
\+ 500-1,400 histomonads/cc
++ 1,500-2,500 histomonads/cc
+++ 2,600-3,500 histomonads/cc

TABLE V

Isolation of Histomonas meleagridis from 1,000 and 2,000 ground Heterakis gallinarum eggs, Experiment 2.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation			
			4	10	16	22
1	0	2	0	0	0	0
2	1,000	4	+	0	0	0
3	1,000	3	0	0	0	0
4	1,000	2	++	++	+	+
5	1,000	1	+	+	0	0
6	2,000	3	0	+	0	0
7	2,000	2	0	+	0	0

0 no growth
\+ 10,000-19,000 histomonads/cc
++ 20,000-25,000 histomonads/cc

similarly inoculated with 1 ml of physiological saline. The surviving 7 poults remained non-symptomatic to 27 days postinoculation.

Two poults were sacrificed on the 27th day postinoculation, one which had been inoculated with a positive culture of isolated histomonads and a second with a negative culture. Necropsy revealed no cecal cores or liver lesions but both had greatly enlarged and inflamed ceca whose contents contained histomonads. Twenty-two days later (49 postinoculation) the remaining 5 poults were reinfected via laparotomy with 28,000 virulent histomonads. When laparotomies were done on these birds, the one given the negative culture had a 'cheesy cecal core'. Within 2 weeks after this reinfection, all 5 poults died of histomonosis.

Experiment 3 was conducted to investigate the possibility of isolating and culturing greater numbers of Histomonas from heterakid eggs and to again verify the isolate as H. meleagridis by producing histomonosis in poults. The results are presented in Table VI. Microscopic examinations revealed histomonads in 9 of 9 tubes after 4 days incubation. All tubes sustained histomonad growth for 14 days without subculturing, however, only 3 of 9 tubes sustained growth for 17 days and 1 for 21 days. This improved growth is probably related to improved technique of the researcher. In Experiment 3, one culture supported as many as 34,000 Histomonas/cc which is greater than any culture in Experiment 2. Both experiments used isolates from 1,000 and 2,000 ground heterakid eggs.

Two, 19-day-old poults were infected via laparotomy and direct cecal injection with 1 ml of culture contents from the 7-day-old culture containing 34,000 Histomonas (Table VI, Tube 2). A third poult was similarly inoculated with 1 ml of physiological saline. All 3 poults remained non-symptomatic to 41 days postinoculation. Thus, these isolates were not identified as

TABLE VI

Improved productivity of Histomonas meleagridis cultures isolated from 1,000 and 2,000 ground Heterakis gallinarum eggs, Experiment 3.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation				
			4	7	14	17	21
1	0	2	0	0	0	0	0
2	1,000	2	+++	+++	+++	++	+
3	1,000	2	++	+	+	0	0
4	1,000	2	++	+	+	0	0
5	2,000	2	++	+	+	+	0
6	2,000	2	++	+	+	0	0
7	2,000	2	++	+	+	0	0
8	2,000	2	++	++	+	+	0
9	2,000	2	++	+	+	0	0
10	2,000	2	++	+	+	0	0

0 no growth
\+ 6,000-19,000 histomonads/cc
++ 20,000-30,000 histomonads/cc
+++ 31,000-34,000 histomonads/cc

H. meleagridis by use of host inoculation. These 3 poults were reinfected via laparotomy with 28,000 virulent Histomonas on day 41 postinoculation. Within 2 weeks after this reinfection, all 3 poults died of histomonosis, necropsies revealed the characteristic circular yellowish depressed liver lesions and necrotic ceca with cecal cores.

Experiment 4 was conducted in order to determine what effect sodium hydroxide-hypochlorite solution may have had on Histomonas within the Heterakis egg. In this experiment the histomonads were released from the heterakid egg by grinding in the Ten Broeck without previous treatment with deshelling solution. Microscopic examinations revealed histomonads in 2 of 9 tubes after 9 days incubation, and only 1 positive tube after 16 days (Table VII). The populations never exceeded 4,400 organisms/cc. Cecal injections of histomonads via laparotomy were done on 3, 2-month-old poults, and 2 of these received a 12-day-old culture containing 4,400 Histomonas/cc, while the remaining bird received physiological saline. At 30 days postinoculation, all 3 birds were sacrificed and necropsies revealed no liver lesions or cecal cores, and microscopic examination of the cecal contents were negative for histomonads. Tubes of complete DeVolt's medium were inoculated with this cecal fluid and microscopic examinations at 3 and 8 days detected no histomonad growth. These data indicated that in the previous experiments, the deshelling solution had no effect on the histomonads within the heterakid egg.

In Experiments 5 and 6, a young culture (48 hours in nutrient broth) of cecal bacteria failed to increase the number of Histomonas in culture when isolated from the heterakid egg (Table VIII). Only 1 of 4 tubes in both experiments had detectable histomonad growth after 13 days incubation. The populations never exceeded 3,000 histomonads/cc.

TABLE VII

Growth of histomonads isolated from heterakid eggs without using deshelling solution, Experiment 4.

Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation			
			9	12	16	21
1	0	2	0	0	0	0
2	2,000	2	+	+	0	0
3	2,000	2	0	0	0	0
4	2,000	2	0	0	0	0
5	2,000	2	+	++	++	+
6	2,000	2	0	0	0	0
7	2,000	2	0	0	0	0
8	2,000	2	0	0	0	0
9	2,000	2	0	0	0	0
10	2,000	2	0	0	0	0

0 no growth
\+ 1,000-1,900 histomonads/cc
++ 2,000-4,400 histomonads/cc

TABLE VIII

Effect of a 48-hour-old cecal bacteria culture on the growth of *Histomonas meleagridis* isolated from eggs of *Heterakis gallinarum*, Experiments 5 and 6.

Experiment	Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation			
				4	9	13	17
5	1	0	2	0	0	0	0
	2	2,000	2	0	0	0	0
	3	2,000	2	0	0	0	0
	4	2,000	2	0	0	0	0
	5	2,000	2	+	++	+	0
6	1	2,000	2	0	0	0	0
	2	2,000	2	0	0	0	0
	3	2,000	2	+	+	+	0
	4	2,000	2	0	0	0	0
	5	2,000	2	0	0	0	0

0 no growth
\+ 500-1,000 histomonads/cc
++ 1,100-3,000 histomonads/cc

Because of the failure of the isolated histomonads to produce liver lesions and death of poults, wing vein injections were done (Experiment 7) in an effort to by-pass the cecal phase of the disease and produce liver lesions and death via a more direct route. Three turkeys injected, via the wing vein, with 35,000 virulent histomonads suspended in complete DeVolt's medium, did not develop symptoms of histomonosis at 3 weeks postinfection. The fourth bird, which received 1,000 artificially isolated histomonads, developed the early symptom of histomonosis of liquid feces which became sulfur-yellow in color at 8 days postinfection. No bloody cecal cores were found in the feces. Diarrhea continued and other symptoms simulating the disease were; weakness, drowsiness, drooping wings, lowered head, ruffled feathers and inappetence. This bird continued to show these symptoms for 30 days postinfection. At this time the 4 birds were necropsied; the liver and ceca were normal and no histomonads were seen in the cecal material. Tubes of complete DeVolt's medium were inoculated with the cecal fluid and examinations at 3 and 8 days postinoculation revealed no histomonads.

Experiment 8 consisted of 4 trials, each an attempt of duplicating the high counts obtained in Experiment 3, but with the use of 48-hour-old cultures of cecal bacteria. The results after culturing this material are shown in Table IX. Microscopic examinations began for all trials on day 4 postinoculation and continued for 27 days. The populations never exceeded 7,700 histomonads/cc. Histomonad growth was detected in only 2 of 4 tubes in trial 1 at 4 days incubation through 23 days. Four of 5 tubes had Histomonas growth after 7 days in trial 2 and after 14 days, only 1 tube had growth for 27 days. Only 2 of 5 tubes in trial 3 and 1 of 5 in trial 4 supported growth.

In Experiment 9 a saline dilution of cecal feces was utilized as the cecal bacteria inoculum to preclude the possibility of losing during incuba-

TABLE IX

Growth of Histomonas meleagridis from various numbers of ground Heterakis eggs, with the use of 48-hour-old cecal bacteria cultures, Experiment 8.

Trial	Tube number	Number of ground eggs from Ten Broeck	Cecal bacteria (drops)	Range of histomonads/cc Days Postinoculation					
				4	7	14	18	23	27
1	1	2,500	2	+	+	+	+	+	0
	2	1,500	2	0	0	0	0	0	0
	3	1,500	2	++	++	+++	+++	+++	0
	4	1,500	2	0	0	0	0	0	0
	5	1,500	2	0	0	0	0	0	0
2	1	6,000	2	0	+	0	0	0	0
	2	3,000	2	0	0	0	0	0	0
	3	3,000	2	++	+	+	++++	++	+
	4	3,000	2	0	++	0	0	0	0
	5	3,000	2	+	+	+	0	0	0
3	1	2,500	2	0	0	0	0	0	0
	2	1,250	2	0	0	++	+	+	0
	3	1,250	2	0	0	0	0	0	0
	4	1,250	2	+	0	0	0	0	0
	5	1,250	2	0	0	0	0	0	0
4	1	1,660	2	0	0	0	0	0	0
	2	1,660	2	+	+	+	+	0	0
	3	1,660	2	0	0	0	0	0	0
	4	1,660	2	0	0	0	0	0	0
	5	1,660	2	0	0	0	0	0	0

0 no growth
\+ 500-1,500 histomonads/cc
++ 1,600-2,500 histomonads/cc
+++ 2,600-3,500 histomonads/cc
++++ 7,700 histomonads/cc

tion in nutrient broth, bacteria that are necessary for histomonad growth. None of the 5 tubes, inoculated with this dilution of cecal feces produced histomonad growth over a period of 4 days to 2 weeks.

In summary, all the heterakid eggs used in the study contained viable Histomonas. This was again verified when eggs from the stock source were inoculated per os into a 4-month-old turkey which developed sulfur-yellow feces on day 8 postinoculation and died 15 days postinoculation, thus, the eggs contained a viable and pathogenic strain of Histomonas.

Morphological Study:

The failure of the histomonad isolates to produce fatal histomonosis in turkeys suggested a need for morphological identification. A preferred method, by some investigators, for the identification of H. meleagridis involves the use of phase-contrast microscopy. Both the isolate and known Histomonas were subjected to phase-contrast and bright-field microscopy. Specimens of H. meleagridis were photographed with bright-field (Fig 1) and phase-contrast (Fig 2) microscopy. Figures 3 and 4 are of the isolate photographed with bright-field and phase-contrast respectively. These data on the morphology when compared with specimens of H. meleagridis from cultures known to produce histomonosis indicated that the isolates were H. meleagridis.

Microscopic examinations of tubes in this investigation revealed the presence of several laboratory contaminants. Fungal mycelia, young fungal sporangia and pollen were commonly contaminating tubes. All of these could be confused with the non-motile, clear hyaline sphere form of Histomonas described by Tyzzer (ibid). Figures 5 (bright-field), 6 (phase-contrast) and 7 (phase-contrast) are photomicrographs of contaminants commonly seen but unidentifiable. Figure 8 is a phase-contrast photomicrograph of a pollen contaminant.

Figures 1 and 2. Photomicrographs taken by bright-field and phase-contrast microscopy, respectively, of a live flagellate Histomonas meleagridis from a 12-day culture. Flagellum cannot be seen. Large round inclusions are starch particles. Approximately 400X.

THIS BOOK CONTAINS NUMEROUS PICTURES THAT ARE ATTACHED TO DOCUMENTS CROOKED.

THIS IS AS RECEIVED FROM CUSTOMER.

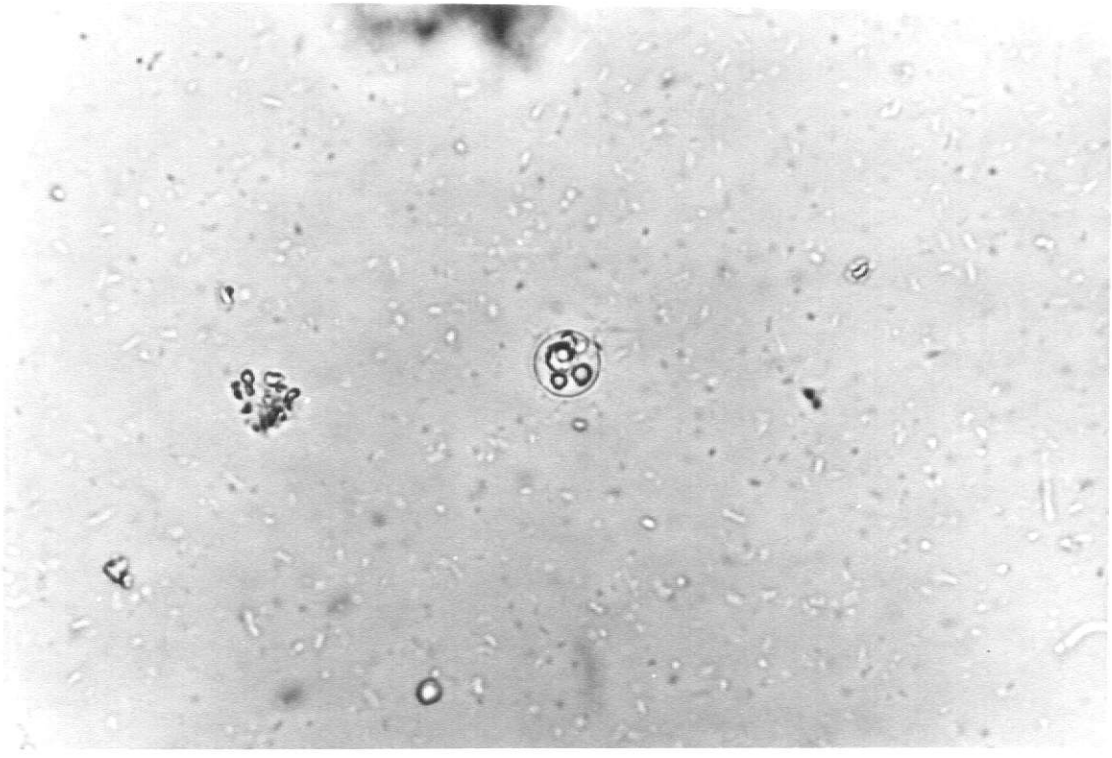

Fig. 1

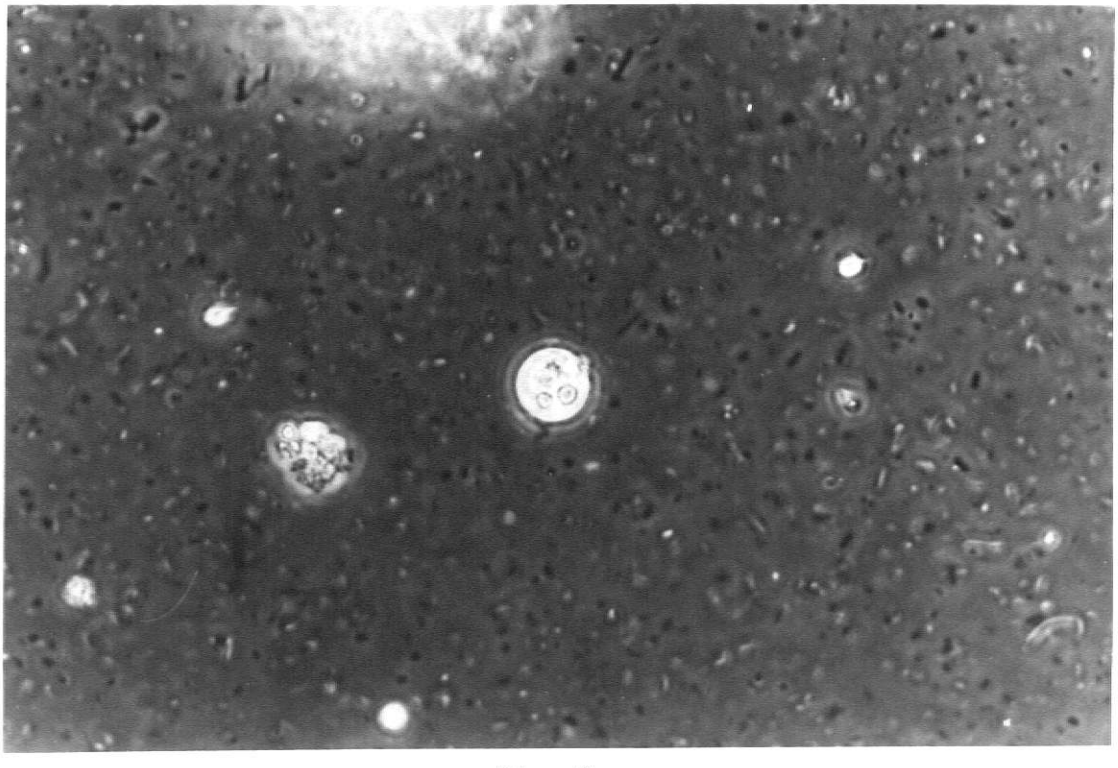

Fig. 2

Figures 3 and 4. Photomicrographs taken by bright-field and phase-contrast microscopy, respectively, of histomonads isolated from Heterakis gallinarum eggs and incubated in vitro for 12 days. Large round inclusions are starch particles. Approximately 450X.

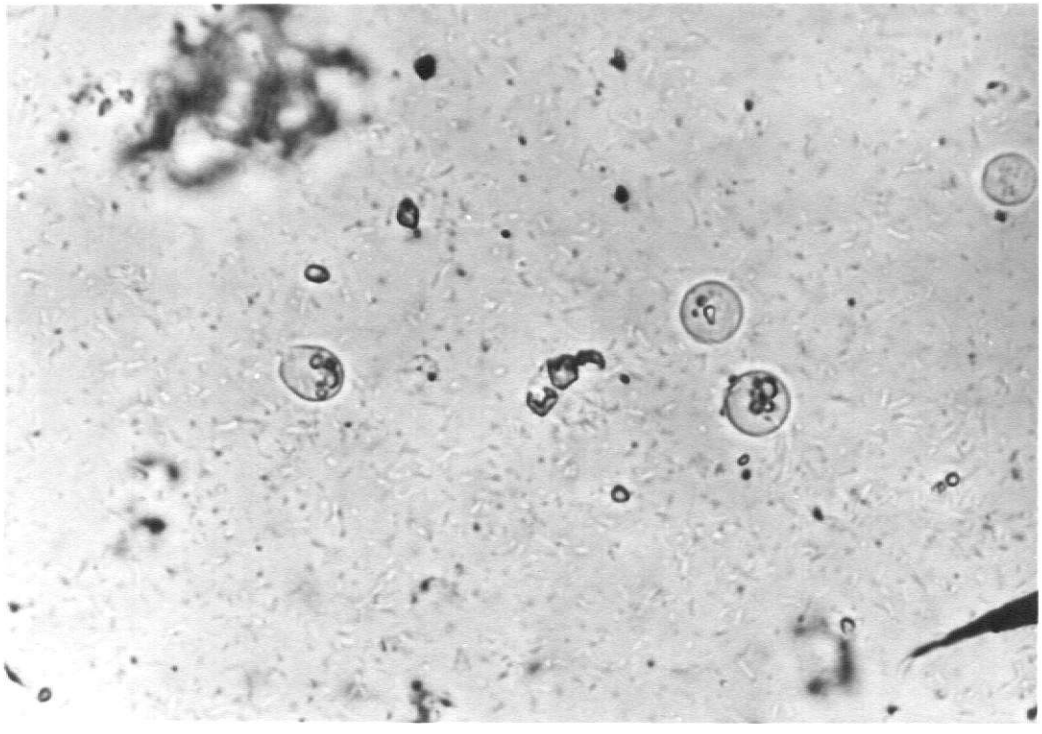

Fig. 3

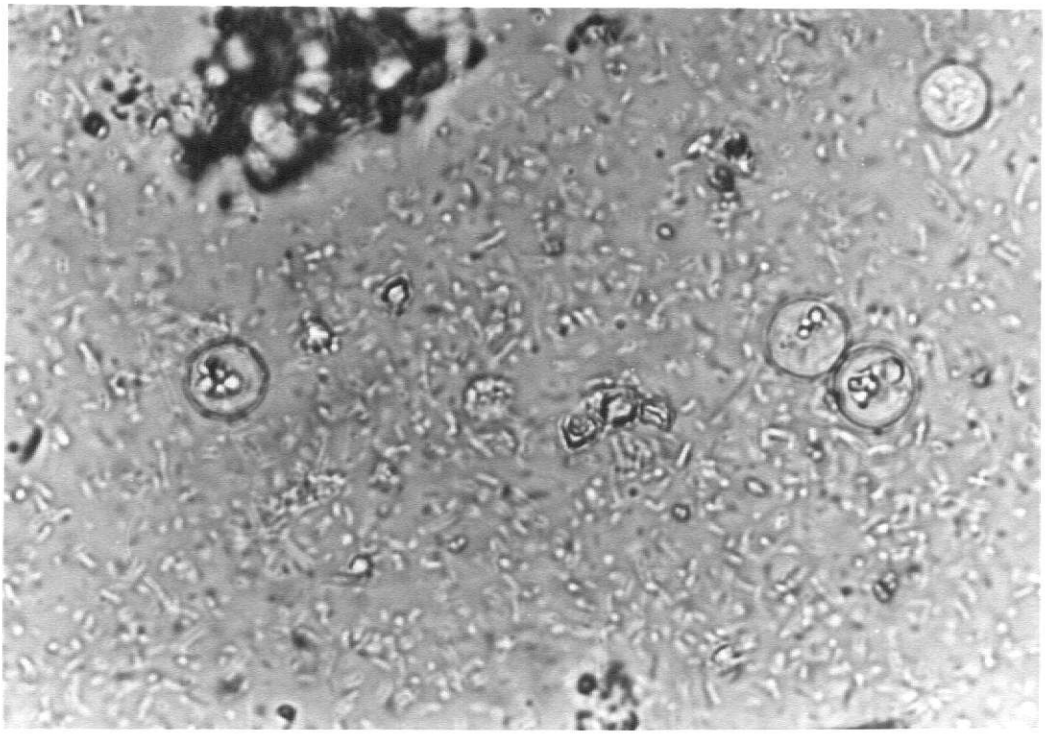

Fig. 4

Figures 5 and 6. Photomicrographs taken by bright-field and phase-contrast microscopy, respectively, of a tube contaminant commonly seen but unidentifiable. Approximately 400X.

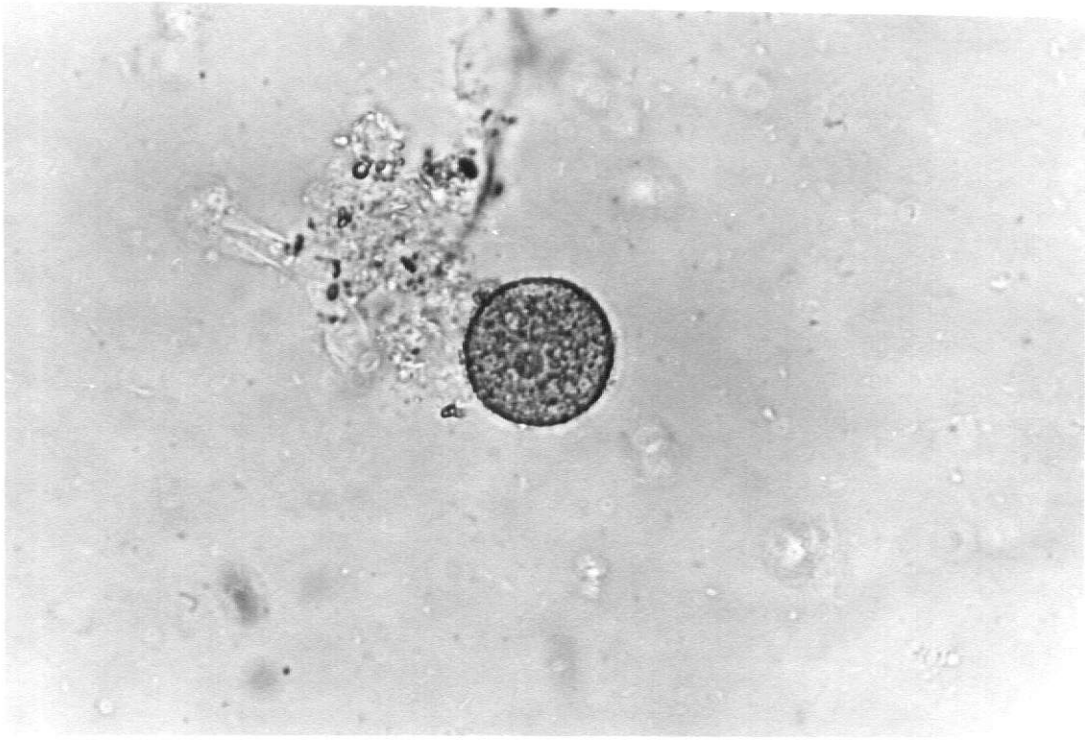

Fig. 5

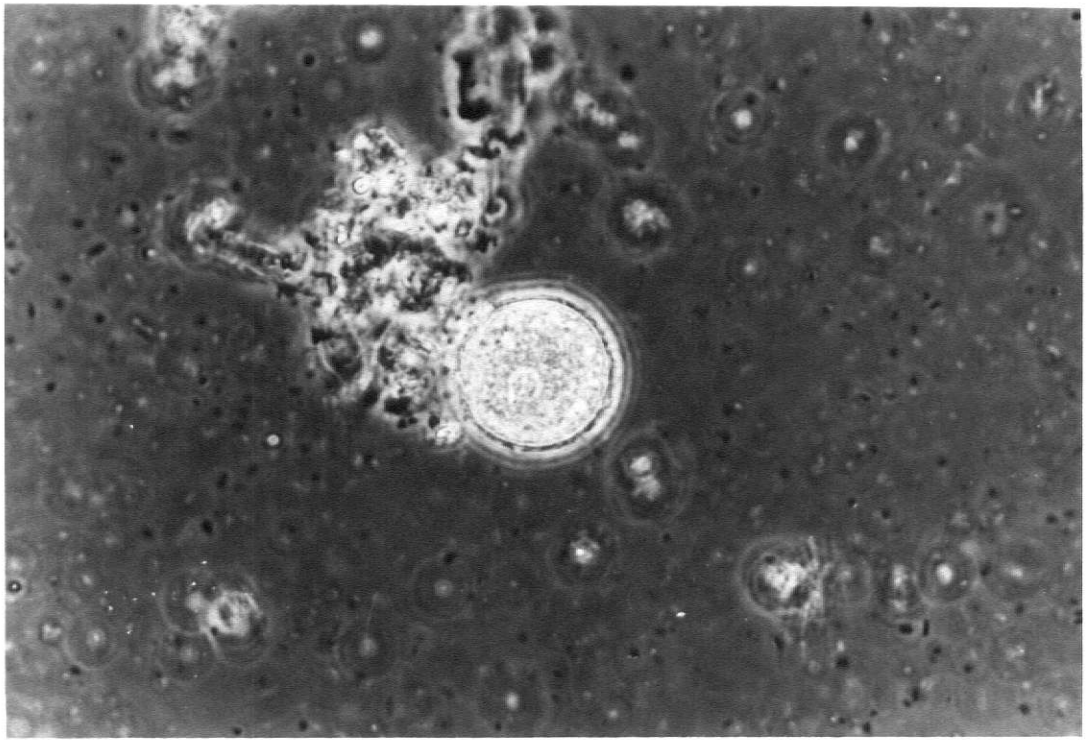

Fig. 6

Figures 7 and 8. Phase-contrast photomicrographs of contaminants of Histomonas cultures, Fig 7 is unidentifiable and Fig 8 is a pollen contaminant. Approximately 400X.

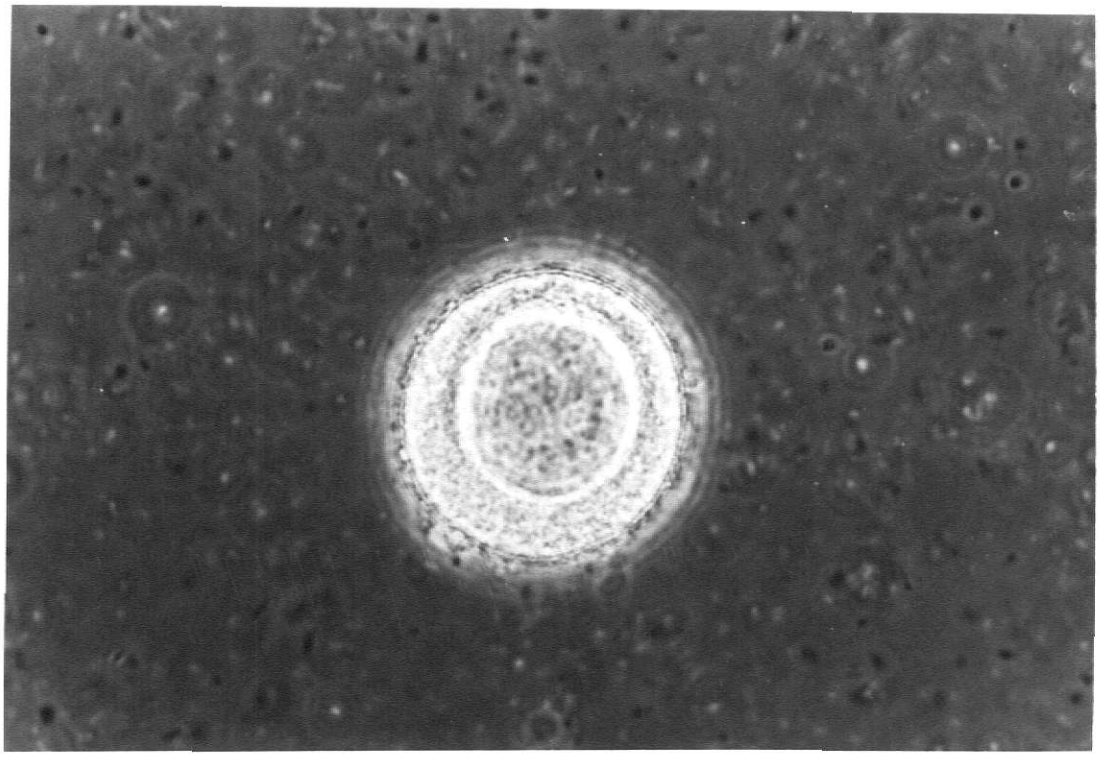

Fig. 7

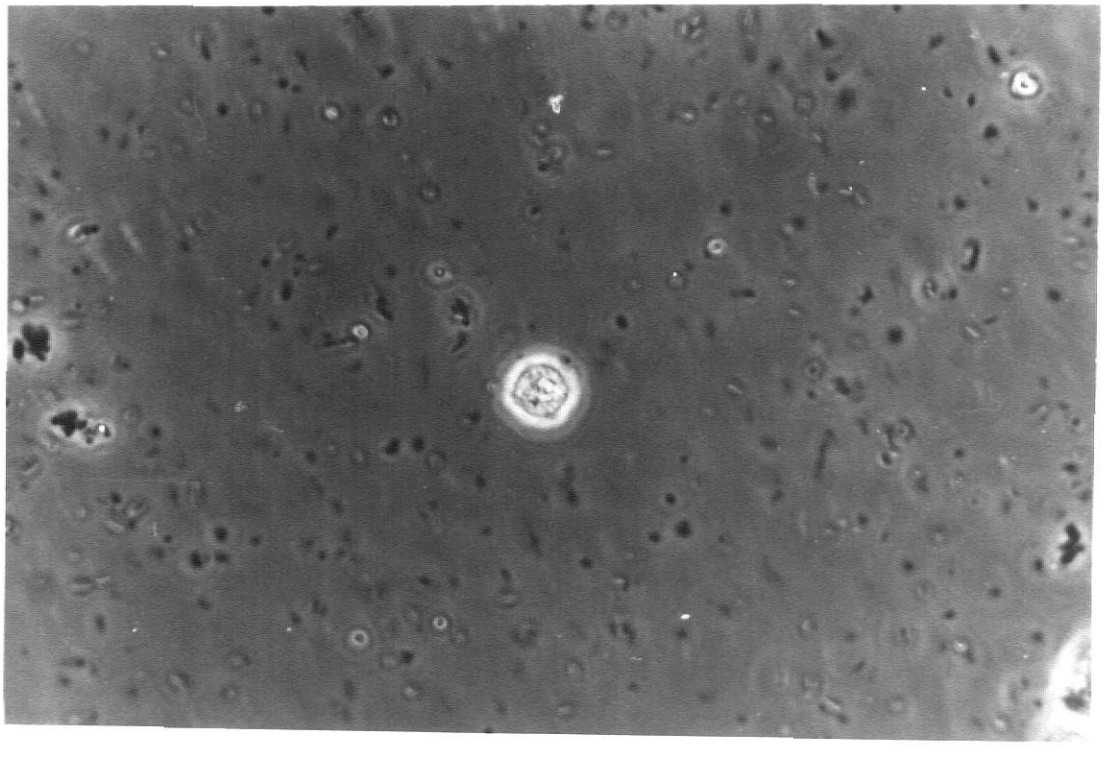

Fig. 8

DISCUSSION

Early studies suggested that histomonads settled to the bottom of culture tubes. Accordingly, Ruff et al, (ibid) used as one source of cecal bacteria inoculum, the bacteria obtained from near the surface of established cultures of histomonads. However, a student in our laboratory (Al-Khateeb) later found as many as 5,000 to 10,000 organisms per ml in the upper portions of the medium (personal communication). Because Histomonas cannot be cultured in nutrient broth, Ruff et al (loc cit) also used an inoculum of cecal bacteria subcultured in nutrient broth to preclude the possibility of transferring histomonads from established cultures.

Using the proper techniques reported by Ruff et al (loc cit) such as sodium hydroxide-hypochlorite solution to remove the shell of the heterakid eggs and cecal bacteria from broth cultures as an inoculum for the histomonad cultures, the presence of Histomonas in the eggs of H. gallinarum was confirmed. Likewise, the study supports the contention by many researchers that the organisms are transmitted in nature via embryonated heterakid eggs.

Histomonads grew in tubes inoculated only with sediment from the tissue grinder-no bacteria added (Experiment 1). This finding is in disagreement with Ruff, et al (loc cit) and demonstrates that Histomonas can be in vitro cultivated in limited numbers in DeVolt's medium without the addition of bacteria.

The number of Histomonas isolated and cultured in Experiments 2 and 3 indicated that it is possible to administer an infective dose of histomonads that will develop into fatal histomonosis. This investigation found that there was a direct relation between the numbers of crushed eggs and the size of the population of Histomonas. Generally, tubes receiving the sediment of 1,000 to 2,000 crushed ova, plus 2 drops of cecal bacteria, resulted in

optimum histomonad growth relative to size of population and longevity.

The birds in Experiments 2 and 3, inoculated via laparotomy and direct cecal injection with 20,000 and 34,000 Histomonas, respectively, should have developed fatal histomonosis at least 70% of the time according to the report of Lund (1955). No fatalities resulted in Experiment 2 but the distended, somewhat turgid and hyperemic ceca developing in 2 birds sacrificed on day 27 postinoculation suggested histomonosis. Lund (loc cit) found that with an injection of 10,000 histomonads, the ceca develop this gross appearance between 7 and 9 days postinoculation, whereas, birds in this study using isolates from the heterakid egg developed this characteristic much later. The distended and firm cecum containing a creamy-white, caseous core found on day 49 postinoculation in 1 of the poults should have occurred at 9 to 10 days postinoculation according to Lund's (loc cit) data.

The reinfection of the poults, in Experiments 2 and 3, with virulent Histomonas was conducted in order to investigate the possibility of protective immunity in birds receiving the apparent non-pathogenic Histomonas. The results of these reinfections showed no significant lapse of time (2 days) between the deaths of the control and challenged birds.

Age of birds as a factor in resistance to histomonosis has been studied by several workers. Curtice (1907) found that 90% of the poults confined in a contaminated area contracted the disease, whereas, only 20% of older birds developed histomonosis. Kendall (ibid) demonstrated that no age immunity occurred in turkeys 7 weeks to 20 months of age when they were experimentally infected either per os with H. gallinarum or per rectum with H. meleagridis.

Perhaps a study could be performed on characteristic liver and cecal changes as the disease progressed, as described by Lund (ibid). Although characteristic cecal changes developed at a much slower rate in birds receiving

the isolate, it is possible that death would result in birds if the numbers of cecal histomonads reached great enough concentrations to allow the disease to progress to termination. It appears that when a few organisms are present, pathogenic qualities are more important than the actual numbers.

Although histomonads could not be seen in several culture tubes in Experiment 2 after 6 separate examinations, they appeared in the ceca of poults 27 days after cecal injection. Accordingly, the present study shows that a supposed negative tube of DeVolt's doesn't necessarily mean the absence of Histomonas but rather a low occult population.

It should be noted that the Nicholas strain of turkey used in these experiments has little resistance to histomonosis (Al-Khateeb, personal communication). Six of 14 poults died in 10 days when rectally infected with histomonads which were continuously passed through turkey poults, i.e. in vivo. Another example of the strains' weakness was demonstrated when histomonads, that had been cultured in vitro for 6 months, killed Nicholas poults in 11 days.

It is not known whether a maturation period of the histomonad within the heterakid egg is necessary before they will be infective. Connell (ibid) discussed the time of release of metacyclic forms of the etiological agent of enterohepatitis in the ceca from H. gallinarum and he postulated that it might be incident to either the second or third larval molts or both. According to Clapham (1933) these molts occur 48 to 96 hours after infection. This problem could be resolved by recovering H. gallinarum larvae from birds at certain intervals after time of infection, crushing them, incubating and culturing this material, and then injecting the cultures into the ceca of susceptible birds. Larson (ibid) failed to produce enterohepatitis in poults by intrarectal injection of crushed H. gallinarum larvae

and neither H. meleagridis nor H. gallinarum were recovered from the cecal contents. However, poults receiving intracecal injections of hatched H. gallinarum larvae and those receiving larvated H. gallinarum eggs per os exhibited typical histomonosis and they harbored H. meleagridis and H. gallinarum. Perhaps the histomonads were present, but did not enter the cecal wall because H. gallinarum were not present to establish a favorable environment for the protozoans. However, it has been demonstrated by researchers that H. meleagridis inoculated intrarectally without H. gallinarum can produce enterohepatitis.

Experiments 4, 5 and 6 were designed to examine the isolation technique used and to discover if any step was involved that altered the virulence or in vitro growth of the isolated protozoan, because when the stock culture of Heterakis eggs were fed per os to poults, death occurred in 2 weeks. In Experiment 4 we eliminated the 3% sodium hydroxide-hypochlorite deshelling solution described by Elliott (1954) but no fatal cases of histomonosis developed in poults inoculated with these isolated histomonads. The low population of Histomonas (4,400/cc) occurring in Experiment 4 could be contributed to the bacterial contamination which could have been eliminated by surface sterilizing the heterakid eggs with a 0.5% solution of peracetic acid according to the method of Doll, et al (1963).

When using a fresh (48 hour) nutrient broth culture of bacteria, in Experiments 5 and 6, very low populations of Histomonas were detected. An older culture of cecal bacteria, subcultured daily over a 2-month period, didn't appear to affect in vitro growth of isolated Histomonas for better cultivation occurred in earlier experiments using it.

The blood phase of the life cycle of H. meleagridis had been postulated but incompletely proven. However, the production of typical lesions in the

liver and the establishment of the parasites in other organs indicates that this protozoan is capable of entrance and survival in the turkey bloodstream. When McGuire and Morehouse (1958) transfused blood from the cecal or mesenteric vein of a diseased bird into the wing vein of susceptible turkeys, typical lesions were produced in the liver with atypical involvement of lungs, kidneys, heart, spleen, pancreas, and proventriculus, but in no instance was the ceca or any portion of the lower intestinal tract involved. No infection was produced when birds were inoculated into the wing vein with whole blood obtained from the wing or heart of the donor bird whose cecal blood produced 33% lethal infection. Clarkson (ibid) described the blood supply of the liver of the turkey and the anatomy of the biliary tract with particular reference to the pathogenesis of histomonosis, and he believed that the hepatic portal vein was the primary vessel transporting Histomonas from the ceca to the liver.

Experiment 7 was conducted in order to bypass the cecal phase of the disease and infect the liver via wing vein injections of histomonads. Why the virulent Histomonas produced no lesions in any organs remains unanswered. The cecal inflammation developed by the bird receiving the isolated histomonads can be attributed to the weakness of the individual and/or any number of organisms such as bacteria, fungi or protozoans. Perhaps intrahepatic inoculation of the isolated Histomonas by liver puncture through the abdominal wall (Harrison et al 1954) would produce liver lesions and eventually the death of the host.

The 4 trials of Experiment 8 confirm and support the data of Ruff et al (ibid) that histomonads can be regularly isolated from embryonated eggs of Heterakis.

The results of Experiment 9 are difficult to interpret because of the

negative results, but it can be hypothesized that if further tests were performed, grinding larger numbers of Heterakis eggs, and using a saline dilution of cecal feces as the bacteria inoculum, positive tubes would be detected. The bacterial flora of freshly voided cecal feces and that taken directly from the cecum by aspiration of nutrient broth, should not be of any significant difference.

Turkey cecal bacteria are necessary in DeVolt's medium to allow favorable growth and longevity of Histomonas cultured in vitro. Using pure cultures of cecal bacteria (Harrison and Hansen 1949), a study could be conducted to determine what kind (genus and species) or group (anaerobe vs aerobe) or combination of groups of bacteria were most favorable to the in vitro cultivation of the histomonad. Tyzzer (1934) found considerable degree of selectivity in regard to food material is shown by Histomonas. In stained films, some organisms were found containing only large bacilli, others minute bacilli, some were loaded with starch particles, and others showed no inclusions whatever. If any particular genus or group of bacteria were selected as a food source and produced satisfactory tube growth, the turkey host could be eliminated as the source of cecal bacteria.

SUMMARY

The presence of Histomonas in the embryonated eggs of H. gallinarum was routinely confirmed by using a sodium hydroxide-hypochlorite solution to deshell heterakid eggs, grinding the deshelled eggs in a Ten Broeck tissue grinder and using cecal bacteria from broth cultures as an inoculum for the histomonad cultures. Thus, the study supports the contention by many researchers that the organisms are transmitted in nature via embryonated heterakid eggs.

There is a direct relation between the numbers of crushed eggs and the size of the population of Histomonas. Tubes receiving the sediment of 1,000 to 2,000 crushed ova, plus 2 drops of cecal bacteria resulted in optimum histomonad growth relative to size of population and longevity. It was found that Histomonas can be in vitro cultivated in limited numbers in DeVolt's medium without the presence of bacteria. Also, a supposed negative tube of DeVolt's doesn't necessarily mean the absence of Histomonas but rather a low occult population.

Histomonas isolated from H. gallinarum eggs failed to kill young turkey poults when inoculated via laparotomy and direct cecal injection - an accepted method for verifying the identity of H. meleagridis. Although no fatalities resulted, clinical symptoms suggested early stages of histomonosis. No protective immunity was produced in poults receiving the apparent non-pathogenic Histomonas.

Neither the sodium hydroxide-hypochlorite solution nor the age of the cecal bacteria inoculum affected the pathogenic quality or in vitro growth of isolated Histomonas.

Attempting to by-pass the cecal phase of the disease, wing vein injections of Histomonas failed to produce liver lesions and death. Only one poult showed symptoms suggesting histomonosis.

When the isolates and Histomonas were subjected to phase-contrast and bright-field microscopy, the isolates were identified as H. meleagridis.

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FACTORS AFFECTING *IN VITRO* CULTIVATION OF *HISTOMONAS MELEAGRIDIS* ISOLATED FROM EGGS OF *HETERAKIS GALLINARUM* AND HISTOMONOSIS IN TURKEYS

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The importance of the eggs of Heterakis gallinarum in the transmission of Histomonas meleagridis is well documented. With the exception of one report, no work has been done on the isolation and culturing of Histomonas from the heterakid egg. In vitro cultivation of Histomonas provides for studying its biochemical requirements and the role of cecal bacteria for its cultivation, survival in culture and pathogenicity.

The presence of Histomonas in the embryonated eggs of H. gallinarum was confirmed. Histomonads were cultured by using a sodium hydroxide-hypochlorite solution to deshell heterakid eggs, by grinding the deshelled eggs in a Ten Broeck tissue grinder and using cecal bacteria from broth cultures as an inoculum. There is a direct relation between the numbers of crushed eggs and the size of the population of Histomonas. Tubes receiving the sediment of 1,000 to 2,000 crushed ova, plus 2 drops of cecal bacteria resulted in optimum histomonad growth relative to size of population (34,000/cc) and longevity (22 days). Histomonas can be in vitro cultivated in limited numbers (500-1,400/cc) in DeVolt's medium without the addition of cecal bacteria which is contrary to the report that histomonads require bacteria in the medium.

Histomonas isolated from H. gallinarum eggs failed to kill young turkey poults when inoculated via laparotomy and direct cecal injection-an accepted method for verifying the identity of H. meleagridis. Although no fatalities resulted, clinical symptoms suggested early stages of histomonosis. When poults were exposed to this apparent non-pathogenic Histomonas isolate and then challenged with virulent histomonads, they died. Thus, the isolate failed to elicit protective immunity.

Experiments were designed to investigate the effectiveness of the isolation technique and to discover whether the technique altered the virulence

of the isolated protozoan. Neither the sodium hydroxide-hypochlorite de-shelling solution nor the age of the cecal bacteria inoculum affected *in vitro* growth of the isolate or its virulence. Because of the failure of the isolated histomonads to produce liver lesions and death of poults, wing vein injections of isolates were done in order to by-pass the cecal phase and perhaps produce liver lesions and death via a more direct route. Again, the birds developed neither liver lesions nor did any die. Only one poult showed symptoms suggesting histomonosis.

The failure of the histomonad isolates to produce fatal histomonosis in turkeys suggested a need for morphological identification. When the isolates and *Histomonas* were subjected to phase-contrast and bright-field microscopy, the isolates were identified as *H. meleagridis*.

Turkey cecal bacteria in DeVolt's medium inhance growth and longevity of *Histomonas* cultured *in vitro*. It is suggested that a profitable study could be done using pure cultures of cecal bacteria such as 1) anaerobes vs aerobes and 2) different genera and/or species in order to determine their suitability in promoting favorable *in vitro* cultivation of the histomonad.