LABORATORY INVESTIGATIONS ON *TRICHOMONAS GALLINAE* WITH EMPHASIS ON DIAGNOSIS

by

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LABORATORY INVESTIGATIONS ON TRICHOMONAS GALLINAE WITH EMPHASIS ON DIAGNOSIS

Introduction

Early in 1973 we became interested in factors which influence pathogenicity and pathology of pathogenic strains of Trichomonas gallinae (Rivolta 1878) especially the very virulent Jones' Barn strain. It was evident that axenic cultures would be useful in such an investigation. Trichomonas-free pigeons should be raised or acquired and these were not readily available. We turned our attention to the diagnostic problems concerned with trichomoniasis in general and T. gallinae infections in particular. The objectives of this investigation then, were to examine possible improvements in axenizing cultures of trichomonads and to examine the application of agglutination reaction in trichomonads as a method of diagnosing immune-pigeons. By immune-challenge procedures it was also anticipated that pathological lesions associated with the J. B strain in pigeons would be described.

Direct wet-film examinations as a method of diagnosing infections are unreliable and the culture method which is usually examined after 24 hours incubation and thereafter daily for 5-7 days detects some infections in which direct smears are negative. However, the culture method does not detect all infections and success depends on the ratio of trichomonads to the contaminating bacteria.

Literature Review

This review of T. gallinae is intended to cover largely the research following Dr. R. M. Stabler's excellent review of 1954 on this

subject. Further, this review deals with papers available to the reviewer at the time of writing. Stabler (1954) dealt extensively on the early nomenclatural problems of T. gallinae. Such names as Cercomonas gallinae and T. columbae etc. are now of historical interest. In the last 15 years research has increased our understanding of the host-parasite interaction of T. gallinae and efforts are in progress to explain these interactions.

Morphology

Using light microscopy, fresh isolates of this organism reveal a pear-shaped outline but with in-vitro cultivation rounded, slender and dividing forms are encountered. Stabler (1954) gave average measurements of fixed and stained flagellate to be 10.5 x 5.2 microns ranging in length from 6.2 - 18.9 μ and 2.3 - 8.5 μ in width. The reviewer agrees completely with Stabler's (1954) observation that the four anterior flagella of the non-dividing organism arising from the basal granule may be lying along the body twisted about one another in an active organism making counting difficult. T. gallinae has an undulating membrane 1/2 - 3/4 of its body-length along the margin of which runs, in a sinuous course, a recurrent flagellum of the same length as the undulating membrane. The mastigont system in this organism is composed of the flagella, the parabasal body, the costa plus its associated granules and the axostyle with paraxostylar granules. The nucleus is anteriorly located close to the kinetosome complex. There is no chromatic ring.

Electron Microscopy: The membrane of the recurrent flagellum appears separate from that of the fin-like dorsal cytoplasmic extension of the

undulating membrane (Mattern, Honigberg and Daniel 1967). The lamella forms a loop within the distal part of the extension. The costa and parabasal filament have a periodic structure while the parabasal body has vesicles similar to the golgi-apparatus of other cells. Mitochondria have not been described for this species but it seems that further studies should reveal them because some enzymes of the Tricarboxylic cycle have been demonstrated (Read 1957). The reviewer feels that the order of fixing this organism for E.M with glutaldehyde and osmium tetroxide should be re-examined. It is known that osmium tetroxide when used before glutaldehyde gave superior preservation of organelles of some hypotrichous ciliates. Shigenaka et al. (1973) claimed that osmium tetroxide may be bound to certain constituents of the cell-membrane which render them resistant to the lytic effects of glutaldehyde.

Cultivation and Biochemistry

Media such as the CPLM (cysteine, peptone, liver, maltose) of Trussell (1946), Diamond's medium TYM (trypticase, yeast extract, maltose) of Diamond (1957), Fluid thioglycollate (Difco or BBL) and almost all media suitable for the cultivation of other trichomonads will within pH limits support the growth of T. gallinae. The CPLM in particular has enough salt concentration to correct for osmotic effects. The Diamond's medium while having much less agar content, which is an advantage, should be modified by increasing the salinity or by using Ringer's solution or any balanced salt solution to dissolve ingredients like in CPLM. The latter modification helps retain the original shape of the organism. A problem with cultivation of trichomonads is the control of associated microorganisms. McEntegart (1952) preferred Seitz filtered medium rather

than heat-sterilized ones in his investigations with T. vaginalis. The technique employed for the isolation and purification of contaminated cultures has been essentially that described by Fitzgerald and Hammond (1954). Further work is needed to simplify the axenization process and it seems that trichomonads that often get entangled in the cotton swab used for sampling can be used to initiate axenic cultures.

Shorb and Lund (1959) in their search for unidentified growth factors for T. gallinae indicated that 3 factors were at least necessary for optimum growth in their synthetic assay medium. Factor R in unpurified ribonucleic acid, factor S in beef serum and factor T in trypticase were reported necessary. The role of albumin, apart from supplying essential nutrients, was to act as a lipid-transporting agent. Limited studies have been carried out on the biochemical components of T. gallinae. There is no reason why some extrapolations cannot be made from similar studies with other species of trichomonads. As Rakoff (1934) rightly observed, "The successful growth of several species of trichomonads in a part of the body which is a foreign habitat for all of them shows a physiological basis of comparison and argues against the specificity of habitat and host which has been claimed for them." Johnson (1960) working with T. foetus and by means of two-dimensional paper chromatography and unidirectional ascending chromatograms has identified the amino acids as alanine, glycine, valine, leucine-isoleucine complex, cystine, lysine, aspartic acid, glutamic acid, proline, tyrosine and phenylalanine. Serine, arginine, threonine and cysteine were only tentatively identified. Mendel and Honigberg (1964) however, analyzed the purified DNA from T. gallinae and showed that this

particular DNA is rich in adenine and thiamine and does display considerable compositional heterogeneity to its molecular structure.

Enzymes of T. foetus were described by Watkins (1959). He mentioned certain enzymes which hydrolyse low-molecular weight substrates known to contain galactosyl, N-acetyl hexosaminoyl and fucosyl structures. Müller and Saathoff (1972) found large quantities of neuraminidase enzyme in cultures of T. foetus. The enzyme was active against almost all the human glycoproteins tested including secretory immunoglobulin IgA. The neuraminidase enzyme is an interesting discovery in T. foetus and further work is indicated to demonstrate this enzyme in T. gallinae and T. vaginalis cultures particularly T. gallinae J.B. strain. Curie et al. (1968) studied the influence of neuraminidase enzyme on the immunogenicity of early mouse trophoblast and Gasic and Gasic (1970) have stated that the enzyme may interfere with some basic mechanisms of reproduction. Müller and Saathoff (1972) suggested that this may well be the same mechanism associated with T. foetus abortion in cattle and if this enzyme is present in the virulent J.B. strain of T. gallinae it will explain its mechanism of invasion through the mucosal barrier and the subsequent invasion of the liver following oral implantation in non-immune columbiformes.

Pathogenesis and Pathology

Perhaps it is pertinent here to define what is meant by the term "strain" when applied to trichomonads. Stabler (1948a) in describing T. gallinae, referred to the particular trichomonads which might be removed from the infected mouth of an individual bird as a strain. It is possible such a bird may harbor more than one physiological strain but

transmission studies have enabled us to define strain as an isolate from a bird with regard to its virulence properties. Diamond (1960) however, quoting Cailleau (1937), said that multiplication of the flagellates through 10 transplants served as criterion of success and that goes to define a new strain in culture. Trichomonads from the upper digestive tract of the chicken have been transmitted by feeding cultures to turkey, bobwhite quail, canary and the English sparrow (Levine et al. 1941). The birds were said to have shown characteristic lesions and the authors described these flagellates as T. gallinae. Stabler and Kilhara (1954) established in trichomonas-free pigeons that single specimens of T. gallinae can produce infections which may lead to death. Stabler (1953) working with the highly virulent J.B. strain in 119 successive trichomonas-free pigeons claimed 100% infection with 114 deaths after 8.2 days post-infection on the average. The above preliminary studies laid the ground work for studies on pathogenicity later reported by Honigberg and Read (1960); Honigberg (1961); Stabler et al. (1964); Honigberg et al. (1964); Frost and Honigberg (1962) and Perez Mesa et al. (1961). Honigberg and Read (1960) reported on virulence transformation of T. gallinae and by the addition of deoxyribonuclease to their homogenate cell-mixture were able to block transformation leading to the suggestion that the enhancement of an avirulent strain by cell-free homogenate of a virulent strain is DNA-dependent. Pathogenicity was investigated by Honigberg (1961) in what he referred to as the "Mouse assay" technique by the statistical comparison of the mean volumes of lesions on the 2nd day to the 14th day produced by various strains of trichomonads following subcutaneous inoculation of stated doses in pure bred mice. In T.

gallinae and T. vaginalis strains, a close correlation appeared between the volumes of the abscesses caused by the parasite strains in the experimental hosts and the degree of pathogenicity observed in the natural hosts infected either naturally or experimentally. This is interesting because it showed that although environmental conditions play an important role in modifying pathogenicity, the basic degree of virulence is an expression of physiological characteristics inherent in the strains of both T. gallinae and T. vaginalis. This fact plus Müller and Saathoff's (1972) isolation of T. foetus neuraminidase serve to point to the suggestion that the pathogenic mechanisms of these flagellates may have the same biochemical basis. Further work in this regard is strongly indicated.

We must be cautioned, however, that continued in-vitro cultivation at 37.5°C of the highly virulent Jones' Barn strain of T. gallinae was shown to be accompanied by gradual loss of pathogenicity for T. gallinae-free or non-immune pigeons (Stabler et al. 1964). The effect on antigenicity is not clear. Some factors which are present in the parasites and which are needed for the expression of pathogenicity, while maintained indefinitely in pigeon to pigeon passage, are gradually lost during frequent division in vitro. Incidentally, suppression of this rapid division by maintenance at sub zero temperature preserves the virulence factors (Stabler et al. 1964). Honigberg et al. (1964) studied the behavior of 2 strains of T. gallinae in trypsin dispersed chick liver cell cultures. They noted that the virulent strain multiplies faster in nutrient medium in the presence of the cell cultures; stimulates greater activity of the macrophages and is not effectively handled by these

phagocytes in which it can multiply causing their ultimate destruction and is found significantly more often within the cytoplasm of the liver epithelial and fibroblast-like cells. The significance of these results should not be overlooked just because these events took place outside some of the immune mechanisms of the host for they do show differences in the behavior of the virulent strain in causing lesions from the mild strains as already indicated by the "Mouse assay" technique.

Frost and Honigberg (1962) studied the histopathology of subcutaneous lesions in mice caused by T. gallinae and T. vaginalis and noted that the pattern of response of the flagellates in their peculiar palisading effect against the inner margin of the inoculation pocket was similar to the arrangement noted in the pharyngeal epithelium of experimentally infected pigeons with J.B. strain of T. gallinae (Perez Mesa et al. 1961).

Frost and Honigberg (1962) therefore proposed a mechanism for the progression of the inflammatory response in these strains as follows: "Multiplication and death of parasites, influx and death of polymorphonuclear leukocytes, lysis of host-tissues with spreading of the lesion and re-multiplication of the organisms established the continuation of the cycle." They noted further that in T. gallinae J.B. strain infections specifically, lysis releases the flagellates from the pocket and edema and the lack of effective fibrosis aid their spread. Perez Mesa et al. (1961) noted that on the second day of infection, T. gallinae J.B. formed a uniform layer on the squamous epithelial surface of the pharyngeal papillae without accompanying inflammation in the underlying connective tissue except near the gland openings and mild mononuclear

reaction of the submucosa. On the third day they described shallow ulcers in the pharynx, focal necroses of the liver accompanied by inflammatory mononuclears and heterophiles. Abscesses were situated in all zones of the liver lobes and trichomonads were difficult to identify. On subsequent days more mucosal ulcerations of the pharynx with massive inflammatory reaction and an admixture of trichomonads and exudate occurred. Liver lesions become larger and appeared partially to be made up of infarct-like areas of necrosis. Grossly, the lesion distribution has been summarized by Stabler (1954) and Nieberle and Cohrs (1970) and occurs in the pharynx, esophagus, crop, sinuses, liver, pancreas, lung, pericardium and peritoneum. Because of the promptness of the necrosis Perez Mesa et al. (1961) ruled out the possibility of hypersensitivity and suggested a possible hepatotoxin for the J.B. strain.

Immunology

Until recently studies on the immunology of T. gallinae have been mainly concerned with protection of the hosts rather than with the antigenic make up of the organism (Honigberg and Goldman 1968). Diagnosis of trichomoniasis using serological techniques is still under investigation particularly T. foetus and T. vaginalis. Morgan (1944) conducted agglutination tests on the normal sera of 24 species of vertebrates, using T. foetus as antigen and among birds, the dove and pigeon had a titer of 1:4 and the rabbit among the mammalian group had the weakest with a titer of 1:4. He concluded that sera of many vertebrates contain agglutinins for the trichomonads. This concept of natural antibodies against trichomonads was later investigated by quantitative agglutination by Samuels and Chun-Hoon (1964) using T. augusta. By natural antibodies

reference is made to those antibodies present in the serum of an animal not known to have been exposed previously to the particular antigen as is true of isohemagglutination system of human blood groups. Heterophil antibodies or antibodies to occult infections have been advanced for the system in trichomonads by the latter authors. This fact Morgan (1944) warned, becomes important when a diagnostic test employing the agglutination reaction is being set up for any species of trichomonads. Immunity to T. gallinae was passively transferred via plasma to non-immune pigeons (Kocan 1970). Stabler (1948a) reported on the protection in pigeons against virulent T. gallinae by infection with milder strains. Thus we can conclude that various strains of T. gallinae have some antigens in common. Dwyer (1972) using the quantitative fluorescent antibody methods established the fact that trichomonads and histomonads share cross-reacting antigens. Structural similarity is said to occur between these organisms from light and electron microscopic findings he stated.

Kocan and Knisley (1970) used the oral immune-challenge procedure to screen 25 pigeons which they established were culture negative for T. gallinae and found that 88% of them were resistant. Kocan and Herman (1970) investigated serum protein changes in immune and non-immune pigeons with various strains of T. gallinae and noted that tissue invasion was necessary to evoke a quantitative change in serum protein concentration as is true with J.B. strain. Immunologic analysis by gel diffusion and quantitative fluorescent antibody methods on T. gallinae were reported by Goldman and Honigberg (1968) and Honigberg and Goldman (1968) respectively. Hayes and Kotcher (1960) devised a direct fluorescent antibody technique for detecting T. vaginalis infections in women

and claimed it was faster and about the same efficiency as the culture method which of course required 48-72 hours of incubation. Documentary efforts using serological methods to diagnose Trichomonas infections in man are those of Trussell (1946); Menolasino and Hartman (1956); Kott and Adler (1961) and McEntergart (1952). There is a need for such efforts to be extended to T. foetus and T. gallinae infections for survey purposes.

Epidemiology

Stabler (1954) dealt extensively with the distribution of T. gallinae geographically and very few additions have been made. There is no data relating to the immune status of any infected population of birds.

Treatment

The use of 2-amino-5-nitrothiazole (enheptin) as an effective drug for canker was investigated by Stabler and Wellentin (1953). Recently conflicting reports appeared in the literature about the role of Hygromycin-B in the treatment of T. gallinae infections. Conrad and Edward (1970) thought that Hygromycin-B could induce trichomoniasis. Kocan (1972) observed that Hygromycin-B actually caused lesions on the mucosa of uninfected pigeon's upper digestive tract. Sannusi (1973 unpublished) agrees with Kocan's findings that Hygromycin-B has corrosive effects on the pigeon's oral mucosa and crop and that death can result from such toxic effects principally by the amount of inflammatory exudation evoked. Suitable assay systems should be employed to determine the toxicity of this drug in birds.

THE MODIFIED MICROAGGLUTINATION TECHNIQUE FOR THE DIAGNOSIS OF T. GALLINAE IMMUNE PIGEONS

I

Materials and Methods

The pigeons were supplied by the Psychology Department here at Kansas State University. The pigeons were obtained from a man in Topeka who raises pigeons and has facilities for nesting. The Psychology Department had information that these birds were free flying around the owner's premises but would hesitate to classify them as wild ones. They were acquired at ages 1-4 months and brought to the Department on a learning experiment so were henceforth raised indoors on commercial pigeon chow and granite grit. Some of the birds received electric shocks, many also lost some weight during the experiment. They were later donated to us for investigations on Trichomonas infections.

Sampling techniques: Samples from the crop, the areas behind the palate, below the tongue, and surrounding oral mucosa were taken with cotton swabs. Samples for cultivation, when desired, were taken with sterile swabs immersed in 2 ml of sterile Locke's solution to which 50,000 units of penicillin and 50,000 µg of streptomycin per ml had been added. Blood for serum analysis was taken by intracardiac bleeding. The pigeon was laid right side uppermost. An area was located mediolateral on a line running at right angles to the anterior tip of the leg when the latter was at the flexed position. The strongest beat of the heart was felt and located at a soft spot close to the rib cage. Using a 20-gauge 1 1/2"

needle and a 10 ml syringe, blood was obtained either from the pumping action of the heart or by suction. Sometimes the heart was missed but the effort was rewarded by drawing dark venous blood from the liver which was adequate for serum preparation. Blood was allowed to clot for 2 hours at room temperature, refrigerated overnight and serum separated by centrifugation. Serum was stored in the freezer compartment of a refrigerator and inactivated at 56°C for 30 min when agglutination studies were anticipated.

Cultivation of T. gallinae: Several media were initially tried for culturing T. gallinae. C.P.L.M. without agar in which the pig liver extract was freshly prepared was investigated. Five hundred grams of ground pig liver and 1 liter of distilled water was boiled for 1 hr, passed through cheese cloth to filter the broth. The broth was made up to 1 liter adding 10 gm of peptone and 1 gm K_2HPO_4 and pH adjusted to 7.00 with 1 N NaOH. About a 1/2 inch liver particle from the cake was placed in each tube with about 8 ml of broth and autoclaved for 20 min at 121°C. The pooled broth plus other ingredients including inactivated horse serum (from Microbiological Associates) was sterilized by filtration through Millipore GS 0.22 μ filters. This medium was discontinued following the successful use of the much simpler, slightly modified Diamond's medium as detailed below: this medium contained trypticase (BBL) 20.0 gm; yeast extract 10.0 gm; maltose 5.0 gm; L-cysteine hydrochloride 1.0 gm; L-ascorbic acid, 0.2 gm; K_2HPO_4 0.8 gm; KH_2PO_4 0.8 gm; oxoid ion agar #2 0.5 gm; distilled water 898.0 ml; sodium chloride 8.0 gm; and brain heart infusion (BHI) 5.0 gm to make 1 liter of medium. The above ingredients were mixed, boiled to dissolve them and

distributed in 16 x 150 mm (Kimax No. 45048) culture tubes covered with Morton culture tube closures with fingers after the pH was adjusted to 7.2. The medium was autoclaved at 121°C and 15 lb pressure for 15 min. The medium was incubated at 37.5°C for 24 hr and re-autoclaved for the second time before final storage at refrigeration temperature. Inactivated horse serum was added just prior to the inoculation of the culture.

Method of axenization: The fact that bacterial contamination poses the greatest threat to successful isolation of trichomonads, efforts were made to simplify the axenization process. Sterile swabs in sterile Locke's solution (2 ml per tube) containing 50,000 units of penicillin and 50,000 µg of streptomycin were used. Diamond (1957) described a procedure in which a cotton swab was first prepared by dipping it into a solution of cellulose acetate in amyl alcohol and allowing it to dry. This treatment was to aid in preventing the entrapment of the flagellates between the cotton fibers. It was recognized that the entrapment of the flagellates could be used to advantage in the axenization process. A lot of the contaminating bacteria with some flagellates of course will pass into the sampling solution. Cultivation using the swab for direct inoculation and subsequent incubation in a culture tube containing the modified medium, for 24 hr, was found to be successful and better than using the solution. After 24 hr incubation at 35°C the swab was discarded and routine subcultures prepared from this isolation. Initially antibiotic additives, 10,000 units of penicillin plus 10,000 µg streptomycin, were added to the Diamond's medium but later, antibiotics could be omitted. Indication for omission of antibiotics can be established by the appearance of a clear zone of medium above the turbid yet

granular growth of organisms at the bottom of the tube or staining for bacteria or other routine sterility tests. Whatever bacteria remained in the swab will be exposed to antibiotics absorbed by the swab and the flagellates that are carried into the Diamond's medium by the swab have better chances of survival. The sampling fluid after the swab culture was prepared was screened by the wet-film examination for trichomonads before being discarded. Strains isolated in Kansas here by this method were labelled L.K. strains, presently $L.K._1$ - $L.K._4$ are available.

The Jones' Barn strain of T. gallinae was isolated from a sterile liver-abscess of an infected susceptible pigeon air-expressed to us by Dr. R. M. Stabler of Colorado University who generously advised on the timing of such isolations. This strain had been in culture here at Kansas since September 1973 and was used extensively in the serological studies.

The Diamond's strain, 3002-L.S. Diamond (NIH)-strain AG (Amherst) of B. M. Honigberg isolated in Dec. 1956 from the mouth, esophagus and crop of Columba livia, originally said to be non-pathogenic was received from the American Type Culture Collection in August 1973. All strains have been kept in the modified Diamond's medium and the J.B. and Diamond's strains were also preserved in liquid nitrogen vapor using sterile glycerine as cryo-protectant.

Preparation of Antigen and Immunization of Rabbits

Both the Jones' Barn strain and Diamond's strain were used to immunize adult white male rabbits. The trichomonads harvested by centrifugation at 1500-2000 rpm for 10 min from a 10 ml 24 hr culture were washed three times using sterile phosphate buffered saline (PBS) and

sterile centrifuge tubes covered with aluminum foil. The washed organisms were resuspended in 5 ml of sterile PBS and the average number of organisms estimated by counting in a hemacytometer with average yields of 3×10^7 living flagellates per ml. Five rabbits were used for the immunization, 2 inoculated intravenously with the J.B. strain and 2 with the Diamond's strain. Preinoculation sera were obtained by intracardiac bleeding. Living antigens (8-10,000 organisms) were inoculated at each dose using the marginal ear vein. When serum samples were needed they were taken by intracardiac bleeding 1 week after the last injection. Weekly inoculations were made, 5 inoculations for the J.B. and 4 for Diamond's strain.

Prior to use in the agglutination reaction all rabbit sera were inactivated at 56° for 30 min to remove non-specific lysis by complement. Trussell (1946) indicated that living flagellates injected intravenously produced higher agglutinin titers than killed organisms and this was established for T. gallinae by Honigberg et al. (1971).

The Modified Microagglutination Technique

The agglutination reactions for trichomonads previous to this have depended on tube-agglutination techniques. The Cooke's Microtiter Equipment (Cooke Engineering Co., Medical Research Division, Alexandria, Virginia) was adapted for use in Trichomonas agglutination reactions. When used, this equipment eliminates the need for slides, coverslips, test-tubes and pipettes. It requires minute amounts of serum. The reaction is undisturbed when scored under an inverted microscope unlike earlier methods. For permanent records the reactions can be photographed using a phase contrast inverted microscope equipped with a camera, e.g.

Nikon Model M as was used here. The Microtiter technique is described below:

(1) Inactivate serum samples 56°/30 min.

(2) Deliver 0.05 ml of sterile PBS in all wells (U-shaped) using the permanent pipette dropper.

(3) Make double dilutions of each serum sample using the diluter pipette. Stir each dilution with the same number of frequency for all dilutions.

(4) Add 0.05 ml of uniformly stirred antigen-preparation (previously quantitated using the hemocytometer) using the pipette dropper. Concentrations of 500-10,000 organisms per well was found to be adequate.

(5) Incubate the prepared dilutions for 2 hours at 37°C. Cover the wells with sealing tape provided with the equipment to prevent evaporation and dust.

(6) Score the reaction using the Inverted Microscope.

(7) By boring a hole of the same diameter as one of the wells in the center of one of the rectangular packing cards for the plastic plates, and attaching this cardboard to the stage of the microscope over the objective with scotch tapes the wells can be slided in position over this hole for specific reading.

(8) (i) Set up a diluent control (PBS).

(ii) Set up a pre-inoculation rabbit serum control. The immunized rabbits provided hyperimmune serum for base-line studies in terms of reaction-rating for the pigeon sera.

Results and Discussion

The axenization process described above may perhaps be suitable for isolating other monoxenic cultures of contaminated protozoal samples. It certainly applies to other trichomonads. Both mechanical and antibacterial measures are necessary for initiating such cultures (Plate 4, Figs. 6 and 7).

The modified Diamond's medium provides enough salinity to correct for osmotic effects and the BHI incorporated provides a sensitive medium for sterility test in addition to enrichment of the original medium. This modified medium was stored for 45 days under refrigeration and was found to be still suitable for maintenance and isolation purposes. This is a desirable characteristic as ordinarily Diamond's medium is good within 7 days storage. Cox and Nicol (1973) working with axenic cultures of T. vaginalis noted that at least 10^5 organisms per inoculum must be present for detection at 24 hr. In general the heavier the bacterial population supported by the medium the poorer the growth of T. gallinae (Diamond 1954) and the fewer the number of trichomonads the less chance for success in getting a positive culture.

Table I shows the summary of the results obtained from direct wet film examinations, swab culture preparations and serum agglutination reactions of the pigeon population sampled. While the direct and cultural examinations revealed the percentage of pigeons harboring trichomonads, the agglutination reactions with the level of antibodies specified for immune pigeons had no bearings to the presence of trichomonads at the time of sera samples because negative pigeons were known to give immune

Table I

Survey Experiments on T. Gallinae in Pigeons

Method	Total no. of samples	% Positive samples
Wet film exam.	170	54.7
Swab culture	117	47.9
Fluid culture	69	15.9
Serum agglutination (Immune titer)	118	44.6 ± 3.1

titers. Immunity in trichomoniasis is of the sterile type persisting over months after loss of infection although eventually it wanes.

Table II shows the level of antibodies as determined by agglutination reactions using T. gallinae J.B. strain as antigen against pre-inoculation rabbit sera. This represented the natural antibody level to trichomonads. Morgan (1944) got 1:4 titer as the level of natural antibodies in rabbits using similar serum dilutions and T. foetus as antigen. Similarly he obtained 1:4 titer as the level of natural antibodies for trichomonads present in pigeons using T. foetus. In the present investigation, using T. gallinae J.B. strain we recorded 1:8 titer as the level of natural antibodies in rabbits against trichomonads.

Table III was included to show the effect of simultaneous dilution of antigen and antiserum of a homologous reaction between J.B. strain and its immune rabbit serum. The experimental set up provided us with a guideline within which results could be expected to be influenced by varying antigen concentration.

Table IV shows a homologous reaction used in following the progress of the immunization and it also provided a guide to the time and number of inoculations needed to reach the hyperimmune state in the experimental rabbits. Similar results were partially obtained for the Diamond's strain (Table V) because on the days of sera collection heart punctures were unsuccessful and therefore the rabbits were left alone to minimize trauma and possible loss during the experimental period.

Tables VI and VII depict heterologous cross-reactions between the various strains of T. gallinae kept in axenic cultures here. It was anticipated that antigenic relatedness could be learned but due to the

Table II

Preliminary Titration of Antigen Against Rabbit Serum Using the Jones' Barn (J.B.) Strain (15 x 10^3 Organisms/Well) (Pre-Inoculation Sera)

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Phosphate buffered saline	-	-	-	-	-	-	-	-	-	-	-	-
Rabbit 2	++	++	++	±	-	-	-	-	-	-	-	-
Rabbit 3	++	++	++	++	-	-	-	-	-	-	-	-
Rabbit 4	++	++	++	+	-	-	-	-	-	-	-	-

Pooled pre-inoculation sera was therefore used as control serum.

Table III

Titration of Various Concentrations of Antigen Against Various Dilutions of Immune Rabbit Serum (4 Inoculations) J.B. Strain of *T. gallinae* as Antigen

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
PBS	-	-	-	-	-	-	-	-	-	-	-	-
Normal rabbit	++	++	++	+	-	-	-	-	-	-	-	-
12,900 organisms/ well	+++	+++	+++	+++	+++	++	++	++	-	-	-	-
3,350 organisms/ well	+++	+++	+++	+++	+++	++	++	++	+	-	-	-
970 organisms/ well	++	++	++	++	++	++	+	+	+	-	-	-
200 organisms/ well	++	++	++	++	++	++	+	+	+	+	-	-

Table IV

Titration of T. gallinae J.B. Strain Against Its Homologous Immune Rabbit Sera at Various Stages of the Immunization Process

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Normal rabbit	++	++	++	+	-	-	-	-	-	-	-	-
Anti J.B. X5 inoculations	+++	+++	+++	+++	+++	+++	++	++	++	++	+	-
Anti J.B. X4 inoculations	++++	++++	++++	+++	+++	+++	+++	+++	+++	++	+	-
Anti J.B. X3 inoculations	++++	++++	++	++	++	+	+	+	-	-	-	-
Anti J.B. X2 inoculations	+++	+++	++	+	+	+	-	-	-	-	-	-

(1.79×10^4 organisms/well)

Table V

Titration of Diamond's Strain Against Its Homologous Immune Rabbit Sera at 3 and 4 Inoculations

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Normal rabbit	±	±	±	-	-	-	-	-	-	-	-	-
Anti-Diamond's X3 inoculations	++++	++++	++++	+++	++	++	+	-	-	-	-	-
Anti-Diamond's X4 inoculations	++++	++++	++++	++++	+++	++	+	-	-	-	-	-

Table VI

Titration of J.B. and Diamond's (D) Strains Against Heterologous Immune Sera After Various Numbers of Inoculations as Indicated by the Preceding Figures

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
J.B. versus anti 3D	±	±	±	±	+	+	-	-	-	-	-	-
J.B. versus anti 4D	+	±	±	+	+	+	+	-	-	-	-	-
D versus anti 5 J.B.	++	++	±	±	±	-	-	-	-	-	-	-
D versus anti 4 J.B.	+++	+++	++	+	+	±	-	-	-	-	-	-
D versus anti 3 J.B.	+++	+++	++	+	+	±	-	-	-	-	-	-
D versus anti 2 J.B.	±	+	+	±	±	-	-	-	-	-	-	-
Normal rabbit versus J.B.	++	++	++	+	-	-	-	-	-	-	-	-

Table VII

Titration of L.K. Strain Against Heterologous Anti J.B. and Anti Diamond's (D) Sera at Various Stages of Immunization as Indicated by the Figures Preceding the Initials of the Strains

	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Normal rabbit	±	±	±	-	-	-	-	-	-	-	-	-
Anti J.B. X5	±	±	±	±	±	-	-	-	-	-	-	-
Anti J.B. X4	++	±	±	±	±	+	+	+	-	-	-	-
Anti J.B. X3	++	±	±	±	±	±	±	±	-	-	-	-
Anti J.B. X2	+	±	±	±	±	-	-	-	-	-	-	-
Anti D X3	+	±	±	±	±	-	-	-	-	-	-	-
Anti D X4	+	±	±	±	±	-	-	-	-	-	-	-

uncertainty of some reactions recorded as ± (agglomeration Plate 3, Fig. 5) we were unable to make definite conclusions.

The reaction scores with the pigeon sera samples were summarized in Tables VIII and IX. Table VIII provided us with a practical working table for assessing immune pigeons within the number sampled. The superiority of J.B. strain of T. gallinae as a group antigen for screening immune pigeons was noted. However, Tables III and VIII showed that the quality of the agglutination reaction as the serum was diluted was important in distinguishing between non-specific or natural antibody reaction, non-protective antibody levels and protective immune titers. For example, the quality of the agglutination with pre-inoculation rabbit sera was never better than 2 plus reaction. For immune rabbit sera after 4 or 5 inoculations, a titer of 3 or 4 plus reaction was recorded over a range of serum dilutions up to 1:32 at least. We can then arrive at the conclusion that a significant immune titer would have to be defined by the quality (reaction score) of agglutination over specified serum-dilution levels.

An immune serum irrespective of origin (pigeon or rabbit) should give a 3 plus or 4 plus reaction (Plate 2, Fig. 5) at 1:1 serum dilution; 2 plus or better score at 1:8 serum dilution and 1 plus or better score at 1:64 serum dilution. On this basis 44.6% ± 3% of the pigeon population sampled were immune, 54.7% were infected using direct examinations and 47.9% were infected as determined from swab culture preparations. It is emphasized that the agglutination reaction described and used to arrive at the above conclusion applies to the antigen specified. The level of significant titer will have to be defined for each antigen or

Table VIII

Experiments with Pigeon Sera Using J.B. Strain of *T. gallinae* as the Antigen of Choice

Score	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Score 0	8/118	8/118	13/118	23/118	44/118	50/118	63/118	78/118	93/118	101/118	107/118	111/118
%	6.8	6.8	11.0	19.5	37.3	42.4	53.4	66.1	78.8	85.6	90.7	94.1
Score 1+	22/118	44/118	44/118	46/118	36/118	39/118	45/118	34/118	22/118	15/118	11/118	7/118
%	18.6	37.3	37.3	39.0	30.5	33.1	38.1	28.8	18.6	12.7	9.3	5.9
Score 2+	33/118	21/118	27/118	35/118	30/118	26/118	8/118	5/118	2/118	2/118	0/118	0/118
%	28.0	17.8	22.9	29.7	25.4	22.0	6.8	4.2	1.7	1.7	0.0	0.0
Score 3 or 4+	55/118	45/118	34/118	14/118	8/118	3/118	2/118	1/118	1/118	0/118	0/118	0/118
%	46.4	38.1	28.8	11.8	6.8	2.5	1.7	0.9	0.9	0.0	0.0	0.0

Table IX

Selected Number of Pigeon Sera Using Diamond's Strain as Antigen (Experimental)

Score	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
0	20/37	24/37	28/37	33/37	35/37	36/37	37/37	37/37	37/37	37/37	37/37	37/37
%	54.1	64.9	75.7	89.2	94.6	97.3	100.0	100.0	100.0	100.0	100.0	100.0
1+	7/37	8/37	6/37	3/37	2/37	1/37	0/37	0/37	0/37	0/37	0/37	0/37
%	18.9	21.6	16.2	8.1	5.4	2.7	0.0	0.0	0.0	0.0	0.0	0.0
2+	8/37	5/37	3/37	1/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37
%	21.6	13.5	8.1	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3 or 4+	2/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37	0/37
%	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

strain of T. gallinae. Kott and Adler (1961) stated that T. vaginalis is not a homogenous species but consists of a number of distinct serotypes which are readily distinguished by simple cross-agglutination and cross-absorption tests although all strains have agglutinogens in common. Teras (quoted by Honigberg and Goldman 1968) was able to place 2 pathogenic strains of T. vaginalis in one antigenic group and a 3rd non-pathogenic strain in another group. We were able to show (Tables VIII and IX) that the antigen of choice here is T. gallinae J.B. and Table IX shows that the Diamond's strain is unsuitable as a standard group antigen perhaps because it was non-pathogenic therefore non-invasive in the host or because of this and the effect of in-vitro passage over the years further attenuating it. The fact that J.B. strain of T. gallinae invades visceral organs especially the liver unlike the less-pathogenic strains which reside in the oral and crop mucosa only, argue in favor of its being more immunogenic. Kocan and Herman (1970) showed that higher β- and γ-globulin levels were detected with J.B. strain and indicated that tissue-invasion was necessary to evoke a quantitative change in serum protein concentration. Although no single serological procedure is sufficient alone to disclose all the antigenic relationships between the strains, the simple cross-agglutination reactions above gave satisfactory indications that agglutinating property is related to pathogenicity in T. gallinae strains. Strain $L.K_1$. despite its freshness was unable to cross-react strongly with immune sera developed against other strains. The pigeon of origin showed no lesions at necropsy. For ease of comparison a reference antigen such as the J.B. strain of T. gallinae should be specified as to the number of

passages in culture and method of subzero storage so that other workers can check available information.

The phenomenon of agglomeration, a weaker form of clumping than agglutination reaction was noticed particularly when working with weak antigens or weak antisera. Plates 1, 2 and 3 are photomicrographs of the reaction scores and scores assessed ± indicated agglomeration reactions in Table VII. Agglomeration is a nuisance and is difficult to score and quantitate (Plate 3, Fig. 5).

Summary of Results

1. A modified Diamond's medium for the isolation and maintenance of Trichomonas gallinae was described. Storage of this medium for 45 days by refrigeration (4°C) does not affect its properties.
2. A simple antibacterial technique was described for initiating cultures of trichomonads and it embodied the use of 10-50,000 units of penicillin per ml and 10-50,000 µg of streptomycin per ml and mechanical separation of trichomonads from bacteria using sterile cotton swabs.
3. A modified microagglutination technique using the Cooke's Microtiter equipment was used to screen sera from a population of pigeons of known history. Using hyperimmune rabbit anti T. gallinae J.B. strain sera, as baseline studies, we were able to define the level of significant immune-titer for these pigeons when living T. gallinae J.B. strain was used as the group antigen.

STUDIES ON THE PATHOLOGY OF T. GALLINAE JONES' BARN STRAIN IN HELMET VARIETY OF PIGEONS (COLUMBA LIVIA). EXPERIMENTAL INFECTIONS USING IMMUNE-CHALLENGE PROCEDURES

II

Stabler (1948a) reported that infection of pigeons by milder strains of T. gallinae could confer immunity and hence resistance to future challenges by more pathogenic strains. Kocan and Knisley (1970) used the oral immune-challenge procedure to screen 25 pigeons and found 88% resistant to T. gallinae J.B. The purpose of the present investigation was to examine the pathology of T. gallinae J.B. strain in the helmet variety of pigeons by challenge infection using oral, intracardiac and intravenous routes. The pigeons were supposedly non-immune and negative for trichomonads using cultural and direct examinations. (Infection does not imply immunity in trichomoniasis as is true of other infectious processes.)

Materials and Methods

Twenty helmet variety of pigeons became available through the Animal Resources Department of Kansas State University. The birds originated from a pigeon fancier who was leaving Manhattan. He claimed that the brown helmets came from Texas. The history of feeding and housing was unavailable but on acquisition they were raised under laboratory conditions on commercial pigeon chow and granite-grit. The birds were all negative for trichomonads on wet-film and cultural examinations over a period of three weeks. Two birds were selected for

each route of inoculation using the very virulent Jones' Barn strain of T. gallinae which had been cultured in modified Diamond's medium for 20 days before challenge infections were initiated. About 3.72×10^6 organisms per ml was prepared in PBS and 2.5 ml given to each bird except the one that was exposed via the intravenous route. Control birds, 2 of which received 2.5 ml cell-free medium which had supported growth of T. gallinae J.B. for 24 hr and 2 others fed 2.5 ml of unused medium orally, were alive throughout the experimental period.

The oral inoculation was done using a 5 ml plastic syringe attached to a 1-2 inch plastic cannula via a needle stump. The intracardiac approach was done using a 5 ml plastic syringe and a 20 gauge 1.5" needle.

The intravenous technique: Feathers were removed from the leg to expose the posterior tibial vein. Using a tuberculin syringe fitted with a 26 gauge hypodermic needle, 0.8 cc of the above concentration of organisms was successfully injected intravenously into only one bird. The smoothness of flow of the preparation and the gradual disappearance of blood in the vein as the fluid entered served as criteria for successful i.v. administration. An approach via the wing vein and entering below the tendon that runs across it was unsuccessful although this technique prevented hematoma.

Results and Discussion

The control birds had no lesions when sacrificed. The oral, intracardiac and the single intravenously inoculated birds died. The birds survived on the average: oral route (7 days), the intracardiac route (5 days) and the intravenous (7 days) post inoculation. The birds

that received oral and i.v. inoculations had similar lesion distributions. Grossly, a few pin-head sized nodules in the pharynx, with hemorrhagic crop mucosa and a pale flabby heart. Air sacculitis and pericarditis with gray fibrinous exudate were noted. The liver had the greatest lesions with small (0.1 mm) to large (0.5 cm) areas of necrosis and sometimes larger coalesced forms of adjacent necrotic areas. All lobes were affected. The lesion distribution with the intracardiac route, however, was interesting. The small pyramidal nodules 0.1 - 0.2 mm, were present on the palatal folds and pharynx with additional lesions on lung tissue close to the heart. The liver lesion at the time of death was localized at the proximal part of the right liver lobe close to the heart. A large necrotic zone was seen instead of the usual uniform distribution over all the lobes.

Histopathology: In the palatal membrane and pharyngeal mucosa (Plate 5, Fig. 8) loss of tissues superficially or large ulcers covered by fibrin and heterophiles. Superficial colonies of bacteria were present in the exudate. The trichomonads, however, were present in the deeper layer of the ulcer. The trichomonads were rounded, stained pinkish with H. and E. and careful examination revealed the presence of undulating membranes in some of them.

Perez Mesa et al. (1961) described in detail the development of lesions in non-immune pigeons and their observation that liver lesions became progressively larger and appeared to be infarct-like areas of necrosis was confirmed. Plate 5, Fig. 9 was a photomicrograph taken from the liver of the bird dosed intravenously. The principal type of necrosis was of the coagulation necrosis type. There were several

circumscribed areas of necrosis involving all and complete lobules within the lobe of the liver but randomly distributed. Many of the hepatic cells within the necrotic areas contained well defined rounded bodies resembling trichomonads. The tissue immediately bordering the areas of necrosis contained some fat vacuoles indicating toxic changes due to the adjacent necrosis.

The lesion caused by T. gallinae has always been described as caseous. This is misleading because grossly the lesions were firm but not cheesy and histopathologically the lesions were mucosal ulcerations in the pharynx and coagulation necrosis of the liver. Death in susceptible (non-immune) pigeons averages 8.2 days (Stabler 1953) and this is too early for caseation to develop. There are strong indications here that the spread of T. gallinae J.B. to the liver following oral implantation in experimental and natural infections is by the hematogenous route since there were similarities in the oral and intravenous type of infections. Cauthen (1936) reported that organisms free of bacteria when introduced into liver, muscle or subcutaneous tissue were capable of maintaining themselves and of causing tissue injury in experimental infections. He however did not establish that the birds were free of trichomonads. Perez Mesa et al. (1961) surmised that the route of spread from ulcerated pharyngeal mucosa to the liver was via the blood. Presently we may conclude from the above experimental findings that T. gallinae J.B. strain gets to the liver of susceptible pigeons by way of the blood or via the lymphatics following the first phase of its pathogenic effects i.e. ulceration of pharyngeal mucosa. The role of secretions from this organism in initiating the ulceration and in promoting

also, the necrotic changes in the liver deserves further studies. The lesion distribution following intracardiac inoculation especially in the liver and other organs in contiguity with the heart, was suggestive of possible invasion of deeper tissues by trichomonads following surface contamination arising from spilling at the site of needle puncture. The mechanism of invasion is postulated to be similar to the mechanism of invasion via the intact oral-mucosa following oral inoculation.

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Plate 1, Fig. 1

Agglutination reaction of living Trichomonas gallinae Jones' Barn strain (established in culture here in Kansas) with a non-immune rabbit serum at 1:16 serum dilution (negative reaction score). Phase contrast Inverted Microscope ~ × 80. Contact print.

Plate 1, Fig. 2

Agglutination reaction of living T. gallinae J.B. strain and an immune homologous rabbit serum + reaction score ~ × 80.

Plate 1

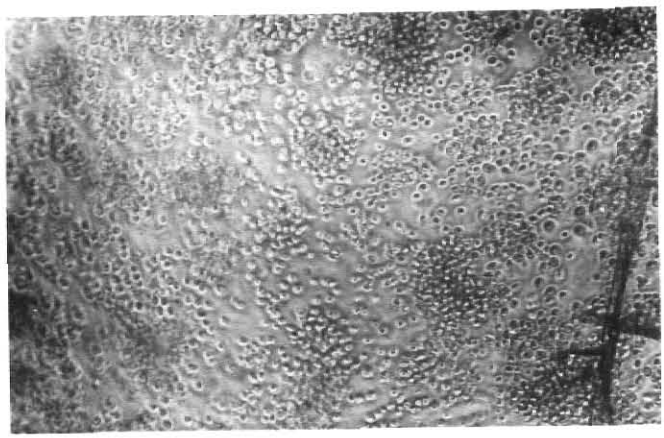

Fig. 1

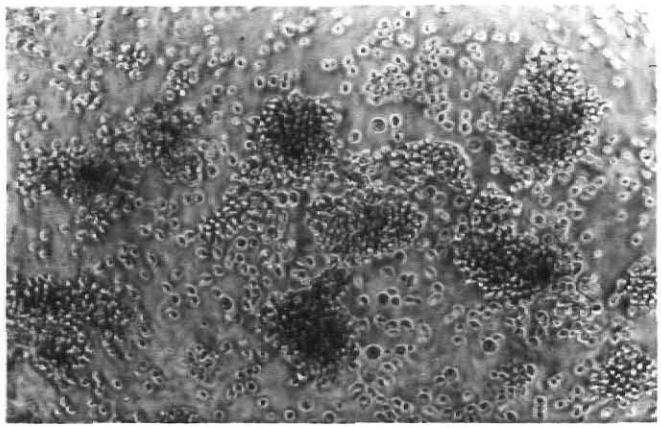

Fig. 2

Plate 2, Fig. 3

Agglutination reaction of living Trichomonas gallinae Jones' Barn strain (established in culture here in Kansas) with an immune homologous rabbit serum. ++ reaction score. Phase contrast Inverted Microscope ~ × 80. Contact print.

Plate 2, Fig. 4

Agglutination reaction of living T. gallinae J.B. strain and an immune homologous rabbit serum. +++ or ++++ reaction score ~ × 80.

Plate 2

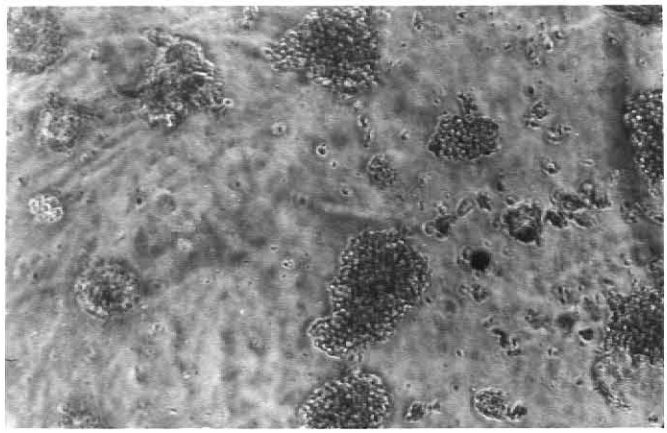

Fig. 3

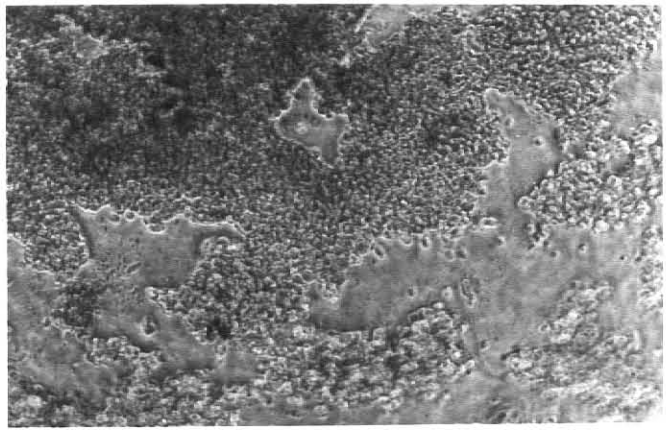

Fig. 4

Plate 3, Fig. 5

Agglutination reaction of living Trichomonas gallinae Jones' Barn strain (established in culture here in Kansas) and an immune heterologous rabbit serum to T. gallinae Diamond's strain at 3 inoculations and 1:4 titer. The reaction score is ± and is here labelled as Agglomeration. Phase contrast Inverted Microscope ~ × 80. Contact print.

Plate 3

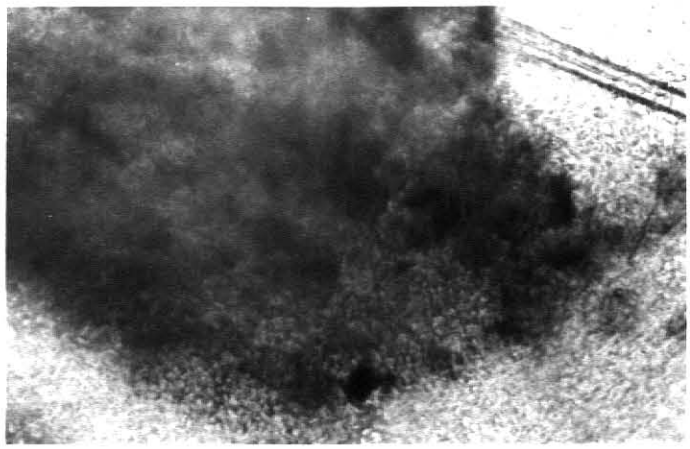

Fig. 5

Plate 4, Fig. 6

Photomicrograph of Trichomonas gallinae Jones' Barn strain in axenic culture. H and E stain - × 320.

Plate 4, Fig. 7

Photomicrograph of T. gallinae J.B. strain in axenic culture at higher magnification. H and E stain - × 432.

Plate 4

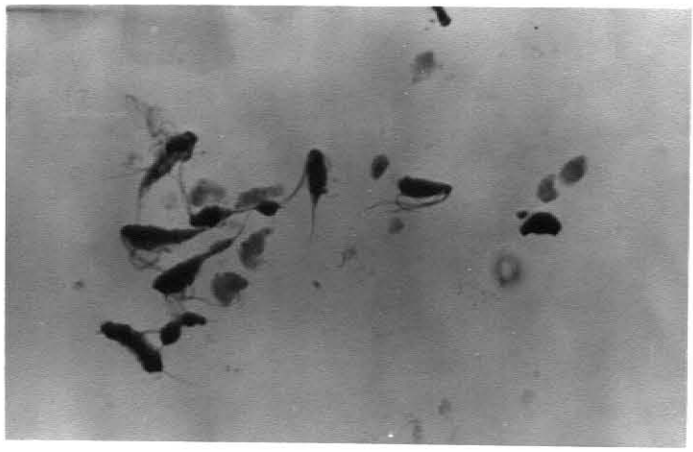

Fig. 6

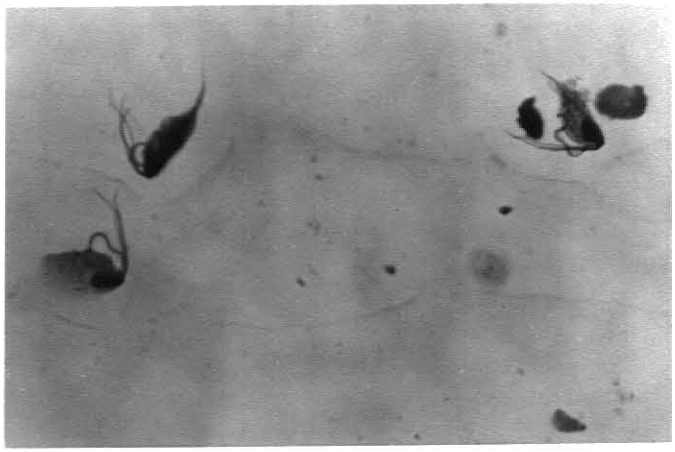

Fig. 7

Plate 5, Fig. 8

Photomicrograph of ulcerative necrosis of the pharynx of non-immune pigeon caused by Trichomonas gallinae Jones' Barn strain after 20 days in culture. H and E stain × 320.

Plate 5, Fig. 9

Photomicrograph of coagulation necrosis of the liver of non-immune pigeon caused by T. gallinae J.B. strain after 20 days in culture. H and E stain × 320.

Plate 5

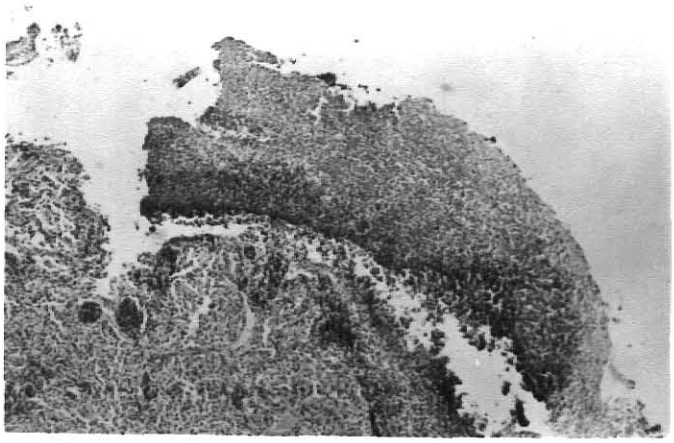

Fig. 8

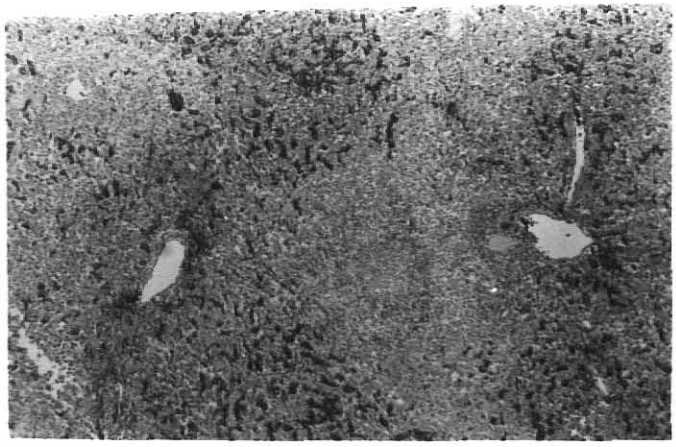

Fig. 9

LABORATORY INVESTIGATIONS ON *TRICHOMONAS GALLINAE* WITH EMPHASIS ON DIAGNOSIS

by

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D. V. M., Ahmadu Bello University, Nigeria, 1971

AN ABSTRACT OF A MASTER'S THESIS

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MASTER OF SCIENCE

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Trichomonas gallinae (Rivolta 1878) is a known pathogen of birds but primarily Columbiformes (pigeons and doves).

An improved method of handling samples from the oral and crop mucosa of pigeons is described and it provides a simple method of axenizing cultures prepared from such original samples. A modified microagglutination technique using Cooke's Microtiter Equipment is presented taking into consideration the fact that natural antibodies to trichomonads are present normally in vertebrates, the significant titer was assessed to depend on the quality of the agglutination at various serum dilutions. Three prerequisites were found to be necessary to describe an immune serum: a 3 plus or 4 plus agglutination score at 1:1 serum dilution, a 2 plus or better score at 1:8 dilution and a 1 plus or better score at 1:64 dilution using the very virulent Jones' Barn strain as antigen. On this basis 44.6% of the pigeons sampled were found to be immune.

Conventional methods such as cultural and wet-film examinations gave 47.9% and 54.7% respectively, these two figures only indicated the percentages by infection. In addition, by immune-challenge procedures using the oral, intracardiac and intravenous routes of inoculation, the predominant lesions in T. gallinae J.B. infections were found to be ulcerative necrosis of the pharyngeal mucosa and massive coagulation necrosis of the liver of non-immune pigeons.