SEROLOGICAL AND IMMUNOLOGICAL STUDIES ON SEVERAL ANIMAL TRICHOMONAS SPECIES

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Hans Jacob Rothenbacher

1955

SEROLOGICAL AND IMMUNOLOGICAL STUDIES ON SEVERAL ANIMAL TRICHOMONAS SPECIES

by

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A Thesis

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the reguirements for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1955

ACKNOWLEDGEMENTS

The author wishes to take this opportunity to express his sincere thanks to Dr. William D. Lindquist of the Department of Microbiology and Public Health under whose inspiration and guidance the present study was made.

The writer also deeply appreciates the financial support of the U.S. Fulbright Commission and the scholarships provided by Michigan State University for the past two years which made it possible for him to conduct this investigation.

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I. INTRODUCTION

Within the last thirty years some disease conditions in man and domestic animals, caused by protozoa of the genus Trichomonas have reached a very high degree of medical and economic importance.

Many workers have been trying to improve the diagnosis and the treatment of these disease-conditions by making use of serological and immunological methods.

The main purpose for this work was to study the serological behavior of three species or varieties of the genus Trichomonas, acquired from the pig and from the turkey, by applying new serological methods and procedures which have evolved in recent years.

Another motive for this study was to confirm the findings of Sanborn (1954), on the serological differences of the two trichomonad species occuring in the nose and the large intestine of the pig.

Last, but not least, the author hopes to stimulate by this study further research, aimed at better diagnostic methods that will eventually lead to a more successful control of the diseases caused by trichomonads.

II. REVIEW OF THE LITERATURE

A. HISTORY

Since Donné's (1836), description of Trichomonas vaginalis from the human female, several other pathogenic trichomonads have been discovered.

Rivolta (1878), found the causative agent of a serious pigeon disease, which is now named Trichomonas gallinae.

Künstler (1888), and Mazzanti (1900), are given credit for having discovered Tritrichomonas foetus in cattle. It was not until several decades later, that these findings gained gradual recognition through the works of Pfenninger (1927), Riedmüller (1928), and Abelein (1929), who established the association of Tritrichomonas foetus with early abortion, pyometra, and sterility in the cow.

In the case of Trichomonas vaginalis, it was Hoehne (1916), who adopted the view that this organism was a causative agent of human vaginitis.

Due to the numerous reports of several non-pathogenic Trichomonas species of man, cattle, and birds, the establishment of one specific Trichomonas species as the etiological agent for a certain disease condition evolved very slowly.

Considerable confusion arose about Rivolta's (1878), discovery, as to nomenclature, and primary pathogenicity of the described organism. After about thirty years of controversy, trichomoniasis of the upper digestive tract and viscera of various birds is now considered as a distinct disease entity (Stabler, 1954).

Quite recently (Hayes, 1955), a serious intestinal disease of chinchillas was found to be caused by trichomonads.

While the pathogenicity of the mentioned species is no longer in doubt, saprophytic and non-pathogenic forms have been reported up to the present from a great variety of animals, ranging from the mammals through birds, reptiles, amphibia, fish, and molluscs, to the insects (Traité de Zoologie, 1952).

In this country it was Emmerson (1932), who first recognized the presence and the wide distribution of T. foetus in cattle. Since that time reports have come from all over the world. Morgan and Beach (1942), in a study of the geographical distribution of bovine trichomoniasis, stated that 23 countries and 35 out of the United States had recognized the disease. Morgan (1946), assumed that trichomoniasis is present in any part of the world where cattle are located.

Because of the improvement in our diagnostic methods, bovine trichomoniasis today has reached its deserved recognition as a disease of high economic importance.

B. SEROLOGY

Numerous workers have attempted to apply serological and immunological methods as an aid for the diagnosis and the treatment of this protozoan infection.

1. Complement-Fixation Reaction:

Riedmüller (1932), was the first to try the complement-fixation test with bovine serum, using the carbolsalt extraction of the peritoneal exudate of Trichomonas-infected guinea pigs as an antigen. While all the non-infected cattle yielded negative results, only a small number of the known infected animals showed a positive reaction.

Witte (1934a), used a carbol-killed trichomonad extract as an antigen for his complement-fixation test. All of the control animals were negative, yet only a few of the infected animals gave very weak positive results. He also reported non-specific reactions from guinea pig sera. In a later publication (1934b), he claimed that his test was specific for cattle sera.

With a method similar to Witte (op.cit.), Endress (1939), tested the sera of about 400 infected and non-infected cattle. In routine herd examinations, and with experimental animals, he frequently encountered a positive reaction from non-infected animals, and a negative reaction from infected animals.

2. Precipitin Reaction:

Besides the complement-fixation test, Zeeti (1940) also tried the precipitin and the agglutination reactions. He was working with the sera of artificially infected guinea-pigs, rabbits, and dogs. His antigen was similar to that of Riedmüller (1932), a saline-extract of the peritoneal exudate of infected guinea pigs. The results obtained did not show a definite correlation between the serological reactions and the infections.

Precipitinogens from the sera of immunized rabbits, and from vaginal washings of infected rabbits were described in further detail by Nelson(1937). His tests showed positive results only in the cases of heavily infected animals.

Robertson (1941), and Morgan (1943), found that the fresh, non-inactivated sera from various domestic animals will kill and

lyse T. foetus in lower dilutions. Inactivation of the sera destroyed the active substance.

3. Agglutination and immobilisation-reaction:

Agglutination of trichomonads by normal guinea pig sera was first mentioned by Witte (1934). This weak normal agglutination was significantly increased in the sera of guinea pigs inoculated intraperitioneally with T. foetus. Yet Witte did not think that this phenomenon was of great value for the diagnosis of the disease in cattle, because of the high level of normal agglutinins in cattle sera. Much later Morgan (1944), published the normal agglutination titres for T. foetus in the sera of 24 different vertebrates, representing Pisces, Reptilia, Amphibia, Aves, and Mammalia.

Zeeti (1940), again confirmed Wittes (1934) findings about the normal agglutinins in guinea pig sera, and also reported the same for dogs. Furthermore, he described a positive agglutination in the serum of rabbits, having been inoculated subcutaneously and intraperitoneally with T. foetus.

By intravenous injection of T.foetus Endress (1939), produced positive agglutinins. After having compared the agglutinations of infected and non-infected cattle, he concluded that the test was of little diagonostic value due to the relatively high titer of normal agglutinins in the cattle serum.

Nelson (1937, 1938), Endress (1939), Morisita (1939), Zeeti (1940), Robertson (1941), Schneider (1941), and Byrne (1942), showed

that rabbit serum had a very weak normal agglutination titer. On the other hand, a strong agglutination titer in normal inactivated equine serum was demonstrated by Endress (1939), Robertson (1941), Schneider (1941), and Morgan (1944).

Byrne and Nelson (1939), compared the titers between the sera of rabbits injected intravenously, with either living or formalin-killed T. foetus suspensions, and rabbits injected vaginally. They found low titers in the latter animals, and high titers in the former group.

Using a formalin - killed Trichomonas suspension, Byrne (1942), described a macroscopic agglutination test. A similar agglutination procedure was described by Morisita (1939), who injected rabbits intravenously with formalin - killed T. foetus. The maximum titers of the sera were attained 25 days after the last injection, and on the 64th day the titers had returned to normal.

Positive agglutinins were also reported by Schneider (1941), in the sera of three rabbits injected intravenously with living or formalin - killed T. foetus.

According to Morgan (1946), all of the previous authors failed to detect the immobilization reaction (Morgan, 1943), because of the relatively small numbers of trichomonads, injected as antigen. It was Robertson (1941), who first mentioned this phenomenon in her agglutination experiments. She injected rabbits with washed and heat - killed cultures of T. foetus, and performed agglutination tests with the immune serum against living culture suspensions of

T. foetus and Eutrichomastix colubrorum. A considerable degree of overlapping was noticed between the two reactions which indicated a common antigenic structure for two different strains of T. foetus, used as antigen. No such group agglutinins were demonstrable in sera produced against Trichomonas columbae. Immobilization was found to occur in the low dilutions of the immune sera (with a high concentration of the agglutinins), characterized by a rounding up of the living organisms, shrinkage of the protoplasm, and non-motile flagella and undulating membrane. The agglutination was described as a ball - like clumping of the trichomonads, which did not decidedly change their normal shape. Loose rosettes were also described, flagella and undulating membrane being motile.

Kerr and Robertson (1941) made use of the agglutination test for the diagnosis of bovine trichomoniasis. Positive titers could never be found in animals with a normal history, or in virgin heifers. They stressed the limitations of both the agglutination and the immobilization reaction, in that, it was not a test on the actual presence of a Trichomonas - infection. It was also stated that sera of heifers, vaccinated intravenously and intramuscularly proved more potent than the sera of normally infected cows, having recently aborted. The diagnosis was made on the basis of a differentiation in the titer and in the kind of the agglutination of the infected animals.

In great detail, Morgan (1943), demonstrated the immobilization reaction in an experiment with eight virgin heifers. Five heifers were injected intramuscularly with living T. foetus. Sixteen in-

jections at three to four day intervals were given in increasing amounts (from five to fifty million), constituting a total number of 350 million living trichomonads per heifer. The first complete immobilization reaction appeared from 16 to 22 days after the first injection in a serum titer of 1:1. The maximum level of 1:128 was reached forty to 76 days after the first injection. The total duration of the immobilization titer varied from five to 105 days in five heifers. A challenging reinjection after 102 days brought all titers up to a positive reaction again. Two heifers injected intravenously according to the same schedule produced slightly higher serum reactions than the ones, injected intramuscularly. Two other heifers, injected intramuscularly with formalin - killed organisms showed relatively lower titers than the heifers, injected with living trichomonads. At no time during the experiment could living trichomonads be recovered from the genital tract of the heifers.

Testing the sera of 51 normally infected cows and eleven bulls, Morgan (op.cit.) could not demonstrate the immobilization reaction. This led him to the conclusion that the degree of anti-body response in the normal infection was low and T. foetus, therefore, not highly antigenic. Thus he considered the reaction of no value as a method for the diagnosis of Trichomonas infection. It should be noted that he did not evaluate the end-titers for the simple agglutination, as did Kerr and Robertson (1941).

In his work on the serological aspects of Trichomonas suis Sanborn (1954), employed living antigen for the inoculation of rabbits. His agglutination procedure included a formalin - killed antigen of a density of about two million organisms per ml, and was similar to that described by Trussell (1947). This method allowed Sanborn (op.cit.) a distinction of the agglutination titers of antisera, produced against different species or varieties of the trichomonads recovered from the pig. He was able to find a slight but definite serological difference between the two Trichomonas species, one taken from the nose and the other from the cecum of the pig.

Pierce (1947) succeeded in demonstrating an agglutinin in the vaginal discharge of four naturally infected heifers. It was not found in two non-infected heifers by the same technique. The appearance of this agglutinin coincided with the disappearance of the living trichomonads from the vaginal or uterine discharge. The agglutination reaction was run with a 1:10 saline- dilution of the mucus diffusion product. There was a correlation between the development of the mucus-agglutinins and the serological response of the heifers, which was demonstrable much later than the mucus agglutination.

The local production of antibodies was confirmed by Kerr and Robertson (1947). They stated that heifers having had an acute Trichomonas infection resisted the reinfection at the time of insemination and calfed at term. This was not the case with heifers

that failed to develop clinical symptoms of an infection upon the first exposure. These authors could also demonstrate the antibodies in the uterine secretions after an artificial sensitization of the uterus by the instillation of the antigen. In an earlier paper, Kerr and Robertson (1946), had reported that the intramuscular vaccination, in spite of a high level of circulating antibodies and a positive skin-reaction, did not protect heifers against the infection with T. foetus, introduced vaginally at the time of insemination.

4. Allergic skin reaction:

All of the early attempts to simplify the diagnosis of bovine trichomoniasis by the allergic skin reaction, have been very discouraging.

In 1944, Kerr published on the intradermal test for bovine trichomoniasis, the application of which is very similar to the tuberculin skin test. However, it can be read after thirty minutes. His antigen represented a complex hapten consisting of the soluble surface component of T. foetus, salted out with trichloracetic acid. This test was reported to correspond well to the serological agglutination test described earlier by Kerr and Robertson (1941).

5. Antigenic structure of the Trichomonas cell:

The recent work by Menolasino and Hartman (1954), throws additional light on the antigenic structure of the Trichomonas cell. These authors were able to separate two major components which were effective in producing high-titer immune responses in rabbits.

They also described a method of observing the antigen - antibody reaction between the living organisms and the immune - sera, produced against the soluble and the insoluble fractions of Trichomonas vaginalis and T. foetus. Twelve to fourteen days after the last injection into rabbits of either the soluble or the insoluble antigen, the immune sera were harvested, inactivated and used for the reaction with the living trichomonads.

Menolasino and Hartman (op.cit.) stated that the insoluble antigen - component produced the following types of antibodies:

a) A specific somatic agglutinin (titer up to 1:5120)

b) A posterior flagellar agglutinin (titer upto 1:640)

c) An antibody complex, causing the rounding, granulation and fragmentation of the living trichomonads. After the fragmentation, a flocculation of the fragments could be observed.

They also demonstrated that the soluble antigen component was found to produce the following types of antibodies:

1. An antibody causing the immobilization and subsequent lysis of the anterior flagella (up to a titer of 1:2560).
2. An antibody causing the sequence of rounding granulation and fragmentation.

In their serological studies these authors showed that T. vaginalis and T. foetus were antigenically indistinguishable. However, it seemed that the two Trichomonas species could be separated by their different titer to the homologous or the heter-

ologous antiserum in the cross-agglutination reactions. The introduction of the agglutination of living trichomonads by Kerr and Robertson (1941), and the modification in the preparation of the antigen for the inoculation of experimental animals reported by Robertson (1941), Menolasino and Hartman (1954), seem to indicate major progress towards a better understanding of the antigenic behavior of trichomonads and the disease conditions caused by them.

III. MATERIALS AND METHODS

A. SOURCES OF TRICHOMONAS SP.

The trichomonads used in this study were the same organisms as isolated by Sanborn (1954). Trichomonas species (nasal isolate) was obtained from the nose of a pig affected with atrophic rhinitis, diagnosed by members of the Department of Animal Pathology.

Trichomonas suis, (fecal isolate), was taken from the cecum of the same animal; Trichomonas gallinarum was isolated from the cecum of a turkey received at the Poultry Diagnostic Laboratory at Michigan State University. A pure culture of Tritrichomonas foetus, originating from the preputial washings of a bull, was kindly supplied by Dr. Donald H. McWade, Dept. of Animal Pathology, Michigan State University.

B. CULTURE MEDIA

For the maintenance of the Trichomonas sp. in stock, C.P.L.M. medium (cysteine HCL, peptone, liver-infusion, maltose) according to Trussell (1947), or fluid thioglycollate broth (Difco) was used.

Sterile horse serum, added to both in a concentration of about four percent, rendered an equally satisfactory growth of all organisms in both media. In the latter part of this study only thioglycollate broth was used in order to simplify the maintenance of stock cultures, as C.P.L.M. - medium was more difficult to prepare.

Another medium producing good growth of the trichomonads, and still not interfering with the procedure of preparing the antigen from the organisms, could be found in the simple brain heart infusion broth (BHI) without agar, to which 0.3% of yeast extract and 0.3% of maltose were added. Sterile, inactivated horse serum in a concentration of about 4% yielded sufficient growth for this purpose. With eleven ml of liquid culture medium in standard (20ml) test tubes, a CO_2 athmosphere as described by Menolasino and Hartman (1954), was found to be unnecessary.

The organisms involved in this study (except T. foetus), had been grown in artificial media for about two years, and thus had adjusted themselves very well to cultural conditions. This was demonstrated by the similar counts of all four Trichomonas species after 48 hours incubation in the same medium. Approximately the same number of organisms having been employed as an inoculum. Counts made with the hemacytometer showed only insignificant variations due possibly to the slight quantitative differences in the inocula.

All cultures could be kept bacteria-free, but occasional contiminants were eliminated by the addition of 1,000 units of penicillin, and 1,000 units of

streptomycin per ml to the described media. This procedure did not have any adverse effect upon the trichomonads.

C. PREPARATION OF THE ANTIGEN

One million trichomonads were seeded into the brain heart infusion medium specified previously. This inoculum rendered a growth of four to eight million organisms per ml after a 48 hours incubation at 37° C.

The test tube culture was mixed thoroughly with a sterile 1ml pipette, and a sample of the trichomonads was counted in a standard hemacytometer. Thereafter, the cells in the tube were centrifuged for ten minutes at 1500 r.p.m., and about nine ml of the supernatant were aseptically removed with a pipette fitted with a rubber bulb. The settled cells were washed in sterile physiological saline and centrifuged for another ten minutes at 1500 r.p.m.; this was repeated three times, the supernatant saline being carefully discarded each time. Using sterile rubber - stoppers, the test tubes were shaken thoroughly after each resuspension in saline.

Next,the sediment of cells was suspended in ten ml of saline in order to count the final number of washed trichomonads in the hemacytometer. Usually about forty to sixty million organisms remained in this last saline suspension. The loss during the washing procedure seldom exceeded twenty per cent of the original total cell count in the BHI - culture.

Quantities of approximately forty million washed trichomonads per test tube were centrifuged at 1500 r.p.m. for ten minutes. After drawing off the supernatant saline, the sediment was resuspended and diluted in ten ml of sterile distilled water. The tubes were then placed in the refrigerator at 4° C for three hours, being shaken vigorously at fifteen minute intervals to prevent settling, and to help a complete rupturing of the Trichomonad cells as described by Menolasino and Hartman (1954). After this three hour period, a microscopic examination was made to check for the complete disruption of the organisms.

In order to obtain two major fractions of this whole Trichomonas antigen, the suspension of the ruptured cells was centrifuged at 2000 r.p.m. for twenty minutes, and the clear supernatant drawn off carefully with a sterile pipette or a syringe. This clear liquid served as the soluble antigen fraction for the injection of the rabbits and chickens. The dosage being measured in correlation to the original number of trichomonads present before the rupturing procedure. For example: Ten ml of the clear supernatant represented the soluble antigen fraction of forty million living Trichomonas cells which were broken up and lysed by the described process.

The insoluble cell fragments remaining in the sediment of the tube were washed and centrifuged once more in distilled water in order to remove all of the soluble cell components. After the supernatant of this last washing was drawn off, the sediment was

diluted with sterile saline up to the desired quantity for the injection into the experimental animals. The dosage of this milky suspension was also correlated to the number of trichomonads present in the test tube prior to the rupturing procedure, and was then used for inoculation as the insoluble antigen component of the whole Trichomonas cell.

Because of this rather crude method of separating two major components of the antigen of the whole Trichomonas cell, it was presumed that the insoluble component still contained a minute fraction of the soluble antigen; however, the clear soluble component could be considered rather pure without containing any "contaminating" insoluble cell substances.

D. INJECTION OF EXPERIMENTAL ANIMALS

Six female, non-pregnant rabbits, approximately six months old, were selected for the first experiment. One week before the experiment, each rabbit had been bled from the heart in order to obtain a small amount of normal serum which, after being inactivated was to serve as a control for the agglutination reactions.

The animals were divided into three pairs: The first pair received the antigens derived from Trichomonas species (nasal isolate); the second pair the antigen components of T. suis, (fecal isolate), and the third pair was inoculated with the antigens of Trichomonas gallinarum. Rabbit "a" of each pair received the soluble antigen component, and rabbit "b" of each pair was inoculated with the insoluble component of its respective Trichomonas species. Six

intravenous injections were made at three to four day intervals. The animals were immobilized by rolling them tightly in a laboratory coat, and the injection given into the external ear-vein under antiseptic precautions. The amount of the antigen was made up to four to six ml for one inoculum. The first injection represented the antigenic components of five million living trichomonads; the second injection equalled ten million, the third fifteen, the fourth twenty, the fifth 25, and the sixth injection contained one of the antigenic fractions of thirty million whole Trichomonas cells of the respective species pertaining to each pair of the rabbits. On no occasion during the injection procedures could any anaphylactic symptoms be noticed in the experimental animals.

Six white leghorn chickens, four months old, and weighing from three to four pounds, were also divided into three pairs and were injected into the brachial vein according to the same schedule as the rabbits. A week prior to the beginning of this experiment, ten ml of blood were drawn from the heart of each chicken in order to obtain a normal serum sample as a control for the following agglutination tests. The amount of the antigen and the time intervals of the injections followed the pattern for the rabbits, except that only the first four injections were made. The amount of one inoculum was made not to exceed four ml per injection.

E. HARVESTING AND PREPARATION OF THE SERA

Ten days after the last injection in the rabbits, twenty ml of blood were drawn aseptically from the heart of each animal.

The blood was allowed to clot at room temperature, and was then placed over night in the refrigerator at 4° C. The next day, after breaking the clot with a sterile pipette, the blood sample was centrifuged at 2000 r.p.m. for two hours. The serum was removed and inactivated by heating in a water - bath at 56° C for 25 minutes, where upon, it was ready for the preliminary serological studies.

Horse and cattle serum for the culture media was prepared in much the same manner, except that it was collected in larger quantities from the jugular vein of these animals.

In order to maintain the level of antibodies in the sera, one week after the first bleeding of the rabbits, all animals were reinjected with their appropriate antigen, corresponding to forty million living trichomonads. Four days later the same amount was again injected. Six days after that all rabbits were bled out in the same manner as described, and sacrificed.

The chickens were bled from the heart one week after the last inoculation, and the sera harvested in like manner as the rabbit sera. No challenging reinjection was applied.

F. AGGLUTINATION REACTIONS

The agglutination reactions were set up in test tube racks, containing ten (12 X 100 mm) serological tubes. To each tube, 0.5 ml of sterile physiological saline was added. The lowest dilution of the antiserum was made up in an extra tube as follows: 0.2 ml of the serum was diluted with 3.8 ml of saline, thus making a 1:20 dilution. From this tube serial dilutions were made down the line,

using 0.5 ml transfers. The serum-saline dilution of the first tube was mixed thoroughly with a 1ml total delivery pipette, and a clean pipette was used for each subsequent serial transfer through tube number nine. Tube number ten served as the saline control for the antigen. For the antisera, dilutions ranging from 1:40 (tube number one), to 1:5120 (tube number nine) were made; in the case of the normal rabbit and chicken sera dilutions were set up from 1:5 up to 1:640, adding 0.2 ml of serum to 0.8 ml of saline in the first tube.

Beginning with tube number ten, a 1:1 ratio of the living antigen was added to the saline control and the serum-saline mixtures; this meant one ml being added to tube number nine and 0.5 ml to all other tubes. The density of the antigen had been adjusted to 500.000 trichomonads per ml. This could generally be accomplished by a 1:10 saline dilution of a brain heart infusion culture, having been incubated at 37° C for 48 hours. Living antigen prepared by this convenient method of direct saline dilution showed equally good results as did an antigen of the same density, washed completely free from the culture medium.

The entire rack of tubes was shaken gently and placed into a 32° C water bath. This temperature was found to interfere less with the viability of the antigen in the saline controls, than did 37° C.

After thirty minutes incubation the first reading of the agglutination was made by microscopic obersavation. A 25 X 75 mm slide was divided into ten squares with a wax-pencil. By means

of a capillary pipette; a small drop of the saline control and the serum - antigen mixtures (approximately 0.015 ml), was drawn from the bottom of each tube, and placed in the proper square of the slide. The drops were allowed to remain for about 3 to 5 minutes at room temperature and were then quickly examined under the low power of a standard microscope for the different types of agglutination. A slide was discarded after 15 minutes due to interference by the air oxygen in the saline control and in the serum dilutions. Therefore, the incubation of the slides for thirty minutes in the moist chamber (instead of the test tube - water bath incubation) was considered as unreliable.

Further readings by the same procedure were made after one hour, two hours, three hours, six hours, twelve hours, and 24 hours incubation. Care had to be taken that the small portion of the antigen-antiserum mixture, to be examined, was taken from the bottom of the tubes in order to get the highest concentration of trichomonads on the slide. This could best be accomplished by drawing a two to three inch liquid column from the bottom of a tube with a capillary pipette and expelling about two thirds of this column. A drop of the remaining fluid was then placed on the proper square of the slide. It was necessary to handle the test tube racks carefully to prevent undue agitation.

The organisms showed the tendency to assemble in the center of the drop on the slide. The agglutination reactions were read serially beginning with the saline control and continuing through

the highest dilution. First, the degree of motility of the living antigen was estimated and recorded. If the motility of the trichomonads present in the saline control was less than fifty percent, the whole test-rack was discarded. This only happened at times when older antigen was used or when the temperature of the water bath rose too high. While observing the agglutination of the living antigen, fresh agglutinations had to be differentiated from agglomerating dead, degenerated, or partly dissolved cells, usually found together with cellular debris, and sometimes encountered also in the saline controls. More than two freshly agglutinated units per microscopic field, each involving at least two cells, were recorded as "one plus" (+). If approximately $\frac{1}{4}$ of the trichomonads in a field were agglutinated, the reaction was recorded as "two plus"; if about $\frac{1}{2}$ of the cells were agglutinated, the reaction was considered as "three plus", and when more than 3/4 of the organisms were involved in agglutinations, the result was noted as "four plus".

G. DIFFERENTIATION OF THE TYPE OF AGGLUTINATION

Since two basically different types of agglutinations were encountered corresponding to the anti-sera produced by the two different components of the Trichomonas antigen, it was felt necessary to describe these phenomena accordingly:

1.) Groups of trichomonads were seen held together by their posterior or trailing flagella, moving their bodies vigorously without necessarily showing a bodily contact. Round and symetrical

rosettes of moving trichomonads could generally be noticed in this type of agglutination which was designated as "posterior flagellar agglutination" (PFA). The organisms showed no tendency to permanently adhere to their bodies or anterior flagella. Thus they were able to move vigorously, in most instances displaying desperate efforts to escape centrifugally from their centerpoint of attachement. In small rosettes, encountered in the higher dilutions of the antisera, this centerpoint was found to be the very distal part of their posterior flagella.

In the lower dilutions of the respective anti-sera, up to several hundred organisms could be observed in ball-like clumps, constantly moving on the periphery, and thus somewhat resembling a colony of Volvox globator (Figure 1).

2.) The second type of agglutination was represented by small or large groups of trichomonads adhering laterally. This intimate adherence did not permit the organisms to move as in the case of the posterior flagellar agglutination. These cellular agglutinations were found to occur in very irregular shapes and never could typically round balls or rosettes be seen. This type of agglutination was therefore designated as "somatic", or "O" - agglutination (OA), (Figure II). Only a very few of the peripherally attached cells were showing some movement of their anterior flagella. The intensity of the reaction in the serial dilutions was likewise also recorded from "Doubtful" ($\pm$) to " four plus", as in the case of the posterior flagellar agglutination. It was also found necessary to record

the degree of immobilisation of the living antigen, occurring in the lower dilutions of this type of agglutination.

H. CROSS AGGLUTINATIONS

Besides the regular, homologous agglutination reaction (i.e. each antiserum produced to the respective antigenic component of one Trichomonas species was reacted with the living antigen of this same organism), heterologous agglutination tests were also conducted by testing each antiserum with the living antigen of all four Trichomonas species involved in this study.

Figure 1.

POSTERIOR FLAGELLAR AGGLUTINATION (P.F.A.)

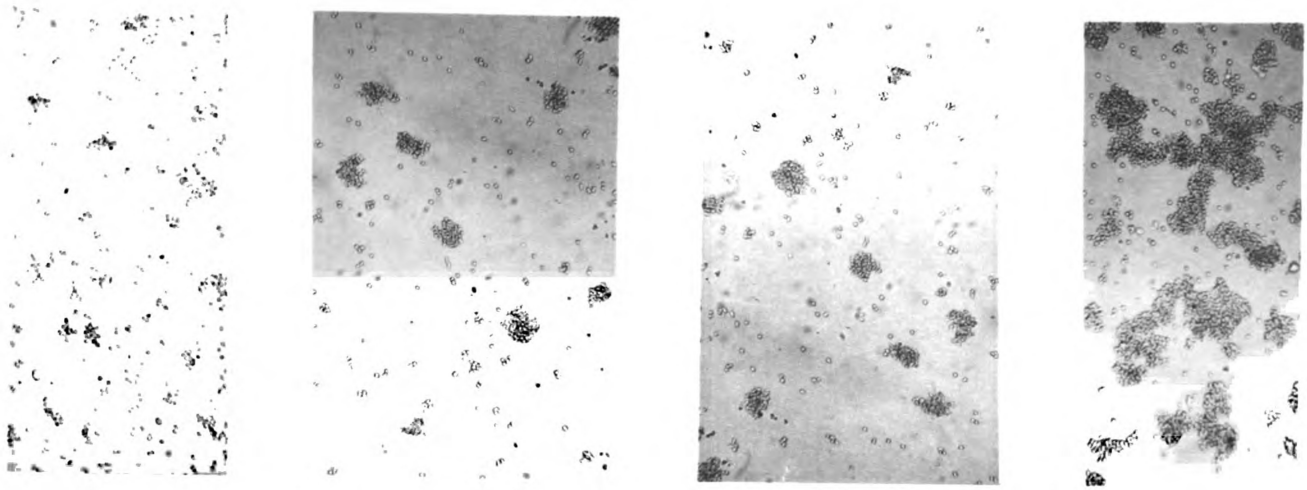
1 2 3 4

Figure 2.

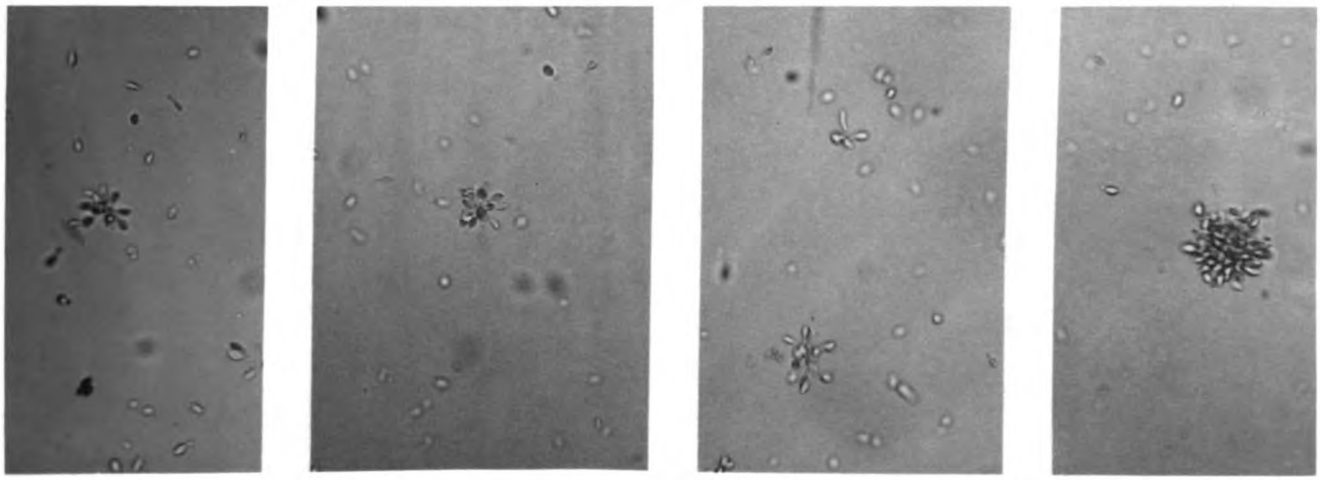
SOMATIC AGGLUTINATION (O.A.)

1 2 3 4

PLATE I.



Somatic agglutination (O.A.)
ca. 100 x



Posterior flagellar agglutination (P.F.A.)

ca. 600 x

IV. RESULTS

A. NORMAL SERUM AGGLUTINATIONS

1. Rabbit serum:

The inactivated serum controls of all rabbits, involved in the study, showed normal agglutination titers for T. species, (nasal isolate) T. suis, (fecal isolate), and T. gallinarum, varying from 1:10 to 1:40. Readings were made after thirty minutes and one hour. It was found that the type of this normal agglutination was predominantly a somatic agglutination. Only the highest dilutions showed some typical P.F.A., and this mainly at the thirty minute reading. A differentiation of the three Trichomonas species according to their normal titers was not attempted.

2. Chicken serum:

The normal agglutination titers of the chicken sera were found to lie between 1:20 and 1:40, as read after thirty minutes and one hour. P.F.A. was predominating in the higher dilutions; only the lower dilutions showed a predominating "O" agglutination of the trichomonads.

3. Horse serum:

Normal inactivated horse serum reacted with the living antigen of the two Trichomonas species from the hog, T. gallinarum, and T. foetus, in a titer from 1:320 to 1:640. The type of agglutination was predominantly a P.F.A., like in the case of the normal chicken sera. No significant difference in titer could be found

in the reactions to the different Trichomonas species.

4. Cattle serum:

The inactivated sera of three virgin heifers, reacted with T. foetus showed a titer varying from 1:80 to 1:320 (average 1:160), as read after thirty minutes and one hour, with P.F.A. predominating in the higher dilutions and O.A. in the lower dilutions. Normal, inactivated beef serum used in the later part of the culturing work, had an average titer of 1:160, as reacted with the different Trichomonas species.

Compared to the results obtained by Morgan (1944) with T. foetus, the normal titers of the rabbits were higher (1:10 to 1:40; Morgan 1:4); also the titers of the chicken sera were slightly above the results of Morgan (1:20 to 1:40; Morgan 1:16). Normal titers of horse sera used for culturing work were found to be considerably lower than the titers observed by Morgan for T. foetus (1:320 to 1:640; Morgan 1:1024, +++). The normal levels for T. foetus obtained from the heifer and from the cow sera compared closely with the findings of Morgan (1:160, Morgan 1:128).

B. ANTISERUM AGGLUTINATIONS AND CROSS AGGLUTINATIONS.

1. Rabbit antisera:

a) Antiserum produced against the soluble cell substances of T. species, (nasal isolate), reacted with the homologous living antigen of the same species, and with the heterologous living antigens of T. suis, (fecal isolate), T. gallinarum, and T. foetus. (Table 1)

Explanation of Tables I through XIII:

P.F.A. = posterior flagellar agglutination only

O.A. = somatic agglutination only

(P.F.A., O.A.) = P.F.A. predominating over O.A.

(O.A., P.F.A.)= O.A. predominating over P.F.A.

\+ = two or more freshly agglutinated units per microscopic field

++ = approximately $\frac{1}{4}$ of the trichomonads involved in agglutination

+++ = approximately $\frac{1}{2}$ of the trichomonads involved

++++ = more than 3/4 of the trichomonads agglutinated

The titers given represent the readings after 30 minutes and one hour. A minimum of three tests for each single reaction was performed in the same manner.

TABLE I

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST THE SOLUBLE CELL FRACTION OF TRICHOMONAS SP. (NASAL ISOLATE).

Living antigen of	Titers
* T.sp. (nasal isolate)	1:1280 P.F.A.
T. suis (fecal isolate)	1:640 P.F.A.
T. gallinarum	1:320 P.F.A.
T. foetus	1:320 P.F.A.

* homologous reaction

b) Antiserum produced against the insoluble cell substances of T. species, (nasal isolate) reacted with the homologous living antigen of the same species, and the heterologous living antigens of T. suis, (fecal isolate), T. gallinarum, and T. foetus.

TABLE II

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST THE INSOLUBLE CELL FRACTIONS OF TRICHOMONAS SP. (NASAL ISOLATE)

Living antigen of	titers Highest dilution	next lower dilution
* T. species (nasal isolate)	1:2560(O.A.,P.F.A.)	1:1280 (O.A.)
T. suis (fecal isolate)	1:640 (O.A.,P.F.A.)	1:320 (O.A.)
T. gallinarum	1:640 (O.A.,P.F.A.)	1:320 (O.A.)
T. foetus	1:640 (O.A.)	

* homologous reaction

c) Antiserum produced against the soluble cell substances of T. suis, (fecal isolate), reacted with the homologous, living antigen of the same species, and with the heterologous living antigens of T. species, (nasal isolate), T. gallinarum, and T. foetus.

TABLE III

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST

THE SOLUBLE CELL SUBSTANCES OF T. SUIS (FECAL ISOLATE)

Living antigen of	titers
* T. suis (fecal isolate)	1:640 P.F.A.
T. species (nasal isolate)	1:320 P.F.A.
T. gallinarum	1:320 P.F.A.
T. foetus	1:320 P.F.A.

* homologous reaction

d) Antiserum produced against the insoluble cell substances of T. suis, (fecal isolate), reacted with the homologous living antigen of T. suis, (fecal isolate), and with the heterologous living antigens of T. species, (nasal isolate), T. gallinarum, and T. foetus.

TABLE IV

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST

THE INSOLUBLE CELL FRACTIONS OF T. SUIS (FECAL ISOLATE)

living antigen of	titers
* T. suis, (fecal isolate)	1:640 (O.A.)
T. species, (nasal isolate)	1:320 (O.A., P.F.A.)
T. gallinarum	1:320 (O.A., P.F.A.)
T. foetus	1:320 (O.A.)

* homologous reaction

e) Antiserum produced against the soluble cell substances of T. gallinarum, reacted with the homologous living antigen of the same species, and the heterologous living antigens of T. species, (nasal isolate), T. suis, (fecal isolate), T. gallinarum, and T. foetus.

TABLE V

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST THE SOLUBLE CELL SUBSTANCES OF T. GALLINARUM

living antigens of	titers
* T. gallinarum	1:1280 P.F.A.
T. species, (nasal isolate)	1:640 P.F.A.
T. suis, (fecal isolate)	1:640 P.F.A.
T. foetus	1:320 P.F.A.

* homologous reaction

f) Antiserum produced against the insoluble cell fractions of T. gallinarum, reacted with the homologous living antigen of the same species, and with the heterologous living antigens of T. species, (nasal isolate), T. suis, (fecal isolate), and T. foetus.

d) Chicken antiserum produced against the insoluble cell fractions of T. suis, (fecal isolate), reacted with the living antigen of the four Trichomonas species.

TABLE XI

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST THE INSOLUBLE CELL FRACTIONS OF T. SUIS (FECAL ISOLATE)

living antigen of	titers highest dilution	titers next lower dilutions
* T. suis, (fecal isolate)	1:1280 (P.F.A.O.A.)	1:640 (O.A.P.F.A.)
T. species, (nasal isolate)	1:320 (P.F.A.O.A.)	1:160 (O.A.P.F.A.)
T. gallinarum	1:320 (O.A.P.F.A.)	
T. foetus	1:320 (O.A.)	

* homologous reaction

e) chicken antiserum produced against the soluble cell substances of T. gallinarum, reacted with the living antigens of the four Trichomonas species:

TABLE XII

CROSS TITRATION OF THE ANTISERUM PRODUCED AGAINST THE SOLUBLE CELL SUBSTANCES OF T. GALLINARUM

Living antigen of	titers
* T. gallinarum	1:1280 P.F.A.
T. species, (nasal isolate)	1:320 P.F.A.
T. suis, (fecal isolate)	1:640 P.F.A.
T. foetus	1:640 P.F.A.

homologous reaction

2. Chicken Antisera

a) Chicken antiserum produced against the soluble cell fractions of T. species, (nasal isolate), reacted with the living antigens of the four different Trichomonas species.

TABLE VIII

CROSS TITRATION OF ANTISERUM PRODUCED AGAINST THE SOLUBLE CELL FRACTIONS OF T. SPECIES (NASAL ISOLATE)

living antigen of	titers
* T. species (nasal isolate)	1:1280 P.F.A.
T. suis, (fecal isolate)	1:320 P.F.A.
T. gallinarum	1:320 P.F.A.
T. foetus	1:160 P.F.A.

* homologous reaction

b) Chicken antiserum produced against the insoluble cell fractions of T. species, (nasal isolate), reacted with the living antigens of the four Trichomonas species.

C. SOME NOTES ON THE GROWTH RATES AND THE LONGEVITY OF TRICHOMONAS SP. IN DIFFERENT CULTURE MEDIA.

1. Growth Rates

Though this study cannot contribute very much to the laborious work of culturing trichomonads done by many research workers, it was felt important to include some data and minor observations which may help future research, in order to obtain a more satisfactory medium for the diagnosis of Trichomonas diseases.

All the culture media described in Materials and Methods, rendered a good growth of the trichomonads; using one million organisms as an inoculum into 11 ml of media with 48 hours incubation at 37° C, the highest concentration of the trichomonads was obtained in the C.P.L.M. - medium, followed by thioglycollate broth and the modified BHI-medium.

It was found that the amount of serum (10% recommended by Trussell, 1947; 20% recommended by Menolasino and Hartman, 1954) could be lowerd to 4% without adverse effects on the cultural work to be done. Also a carbon dioxyde atmosphere of 10%, as reported by Menolasino and Hartman (1954), could easily be omitted.

Due to the relatively high normal titer of horse serum for all Trichomonas sp., beef serum proved even more advantageous and rendered equally high or higher counts under the same conditions.

The following average counts per ml could be determined after 48 hours incubation at 37° C, with an initial inoculum of one million organisms into 11ml of medium:

Explanation for tables XIV and XV:

Numbers represent millions per ml

B.H.I.Y.M.S.A. = brain-heart infusion, plus 0.3% yeast extract, plus 0.3% maltose, plus 4 % horse serum, plus antibiotics.

Thio SA. = Brewer's thioglycollate broth, plus 4% horse serum, plus antibiotics.

TABLE XIV

AVERAGE COUNT IN MILLIONS PER ml AFTER 48 HOURS INCUBATION

Medium	Trichomonas sp.			
	T. species, (nasal isolate)	T. suis, (fecal isolate)	T. gallinarum	T. foetus
B.H.I.Y.M.S.A. (30 counts)	7.01	7.46	6.81	5.77
Thio. S.A. (20 counts)	7.49	8.11	8.60	7.42

TABLE XV

AVERAGE COUNTS IN MILLIONS PER ml AFTER 72 HOURS INCUBATION

Medium	Trichomonas sp.			
	T. species (nasal isolate)	T. suis (fecal isolate)	T. gallinarum	T. foetus
B.H.I.Y.M.S.A. (10 counts)	5.76	4.28	3.71	5.01

The speed of growth was fastest in the thioglycollate and in the C.P.L.M. medium, and slower in the modified B.H.I. medium. The peaks in growth were usually reached after 36 to 48 hours incubation. After that time there followed a quick dying off process of the trichomonads, which was most pronounced in the thioglycollate broth, and less in the modified B.H.I.; C.P.L.M. medium standing in between.

All media had an initial pH of 7.0. As reported by Trussell (1947), and confirmed by Sanborn (1954), the dying of the organisms in the media went hand in hand with a lowering of the pH. This led Trussell (op.cit.) to the conclusion that the trichomonads are killed by the acid production in the medium. The observation of Sanborn (op.cit.) could be confirmed in this study, that the acid production by the trichomonads did not have a primary effect on the killing of the organisms, though it showed a definite correlation with the dying rate of the trichomonads in vitro. A 63-day old C.P.L.M. medium with a pH of 4.5 and 5.0 in one instance, rendered the same growth rates as did fresh medium with a neutral pH.

2. OBSERVATIONS ON THE LONGEVITY OF THE TRICHOMONAS SP.

It was reported by Switzer (1951), and Sanborn (1954), that Trichomonas cultures lasted longer if kept at room temperature. Sanborn (op.cit.) stated, that C.P.L.M. cultures kept for five to seven days at 37° C incbation. In the present study this was also found to be true for the modified B.H.I. medium, while in the thioglycollate broth after 5 days at 37° C usually no living

organisms could be found. According to Sanborn (1954), thioglycollate and B.H.I. medium did not support the growth of the trichomonads from the hog, or T. foetus. In thioglycollate (without serum?), he found them alive for two to three days without multiplication; in B.H.I. semisolid (without serum?), the organisms were reported to stay alive for about fifteen days without multiplication. In this work, however, the hog trichomonads and T. gallinarum could be kept alive in fluid thioglycollate medium without serum for at least 7 days at 37° C. Using an inoculum of 10.000 trichomonads per ml, there was a thirty to forty fold multiplication after 48 hrs 37° C. For fluid B.H.I. with 0.3% yeast extract, but without serum or agar, the multiplication factor was three to five fold after the same time.

In longevity tests at room temperature with the complete media, containing about 4% of horse serum, all of these trichomonads could routinely be kept for about ten days in thioglycollate and B.H.I. medium after an initial incubation of 24 hours at 37° C. In C.P.L.M. medium the organisms could be found alive after 23 days. This compares well with the results obtained by Sanborn (1954). On one occasion T. species. (nasal isolate) was still living after 32 days in C.P.L.M. medium.

In high test-tubes (15 X 200mm), with a liquid column of the medium of about 140 mm (18ml volume), T. gallinarum and the two hog Trichomonas sp. kept mobile for about thirty days in the fluid B.H.I. medium with 4 % horse serum. The same Trichomonas sp.

were positive after 60 days in the thioglycollate medium (plus 4% serum), and T. gallinarum and T. species, (nasal isolate) were found to be still alive after 103 days under the same conditions.

In an accidental finding, T. species, (nasal isolate) showed motile trichomonads after having been stored in the ice box at 4° C for 42 days. In another instance, the same species was found alive in B.H.I. semisolid (4% serum) after a storage of fifty days at room temperature.

V

DISCUSSION

Since this study was dealing with well adapted, two year-old laboratory cultures (except T. foetus), the minor differences in growth rates, speed of growth and longevity were not thought to be significant enough to characterize a separation of the different Trichomonas sp. on the basis of these findings.

Though the serological procedures were modified and different from those of previous workers, the titers obtained especially in the homologous reactions compared closely to the earlier reports.

As there is no standard procedure for living Trichomonas agglutinations worked out at the present time, the results of different workers have to be evaluated accordingly. Also the human factor in judging the titers must be considered.

It was felt that the establishment of an agglutination titer for normal or immune sera of domestic animals with a living protozoan like Trichomonas is a process which differs in some respects from the agglutination of formalinized bacteria. In observing the slide-agglutinations and in checking the reactions for an extended time, biological and physical factors are liable to cloud the picture,and lead to wrong interpretations. The agglutination incubation time was therefore standardized to not more than two hours and the results of later readings were not evaluated in this work.

Further research is necessary to study the degeneration processes under the adverse environmental conditions created by the

agglutination procedures. A more thorough knowledge about the phenomenon of spontaneous agglutination would be desirable. This phenomenon was occasionally seen under the microscope in samples from culture media as well as in the saline controls, after having been exposed to the air for a certain time. Because of its close resemblance to the P.F.A., special care had to be taken in the reading of this type of agglutination.

Contrary to the report of Sanborn (1954), that there was: "little or no evidence of a group antigen among the two Trichomonas species from the hog and T. foetus," the results of this study showed a strong evidence of a common group antigen among all four Trichomonas sp. used. The present isolate of T. foetus, which was not the same culture as used by Sanborn (op.cit.), was acting as a control. This points out the antigenic similarity of all Trichomonas species involved.

While Menolasino and Hartman (1954), in similar studies on T. foetus and T. vaginalis came to the conclusion that these two organisms were antigenically indistinguishable, Sanborn (1954), stated that :"The agglutination experiments showed conclusively a definite antigenic difference between the two Trichomonas forms used from the pig."

According to the results of this work neither of the foregoing statements can be agreed upon completely.

For both antisera produced against the soluble and insoluble antigen fractions, the cross agglutination titers between the two

hog Trichomonas forms compared to the findings of Sanborn (op.cit.), though the differences in titer were not as striking as in Sanborns work. However, it was felt that these differences were significant and consistent enough to justify a differentiation of the two Trichomonas species from the pig, as stated by Sanborn (op.cit.).

It was also found that the titers obtained from the homologous and heterologous reactions of all Trichomonas sp. studied, resembled closely the titers given by Menolasino and Hartman (1954), for the somatic agglutination of T. foetus and T. vaginalis.

All the culture media used in this study (C.P.L.M., thioglycollate, B.H.I. modified, and B.H.I. semisolid), rendered satisfactory growth rates. This was somewhat in contradiction to the results of Sanborn (1954), who reported that thioglycollate and B.H.I. did not support the growth of his Trichomonas sp.

Continuous transfers of the trichomonads were possible in all of the media described above. The serum content in the media could be lowered to 4% without any detrimental effects. Special precautions like a 10% CO_2 athmosphere according to Menolasino and Hartman (1954) were found to be superfluous.

The average counts after 48 hours incubation are self explanatory and lie above the averages reported by Sanborn (1954); about one million organisms were used for the initial inocula per test tube, in comparison to Sanborn (op.cit.), who obtained the best results with inocula of 100,000 to 200,000 into 10 ml of medium.

Experiments with the longevity of the trichomonads were not routinely performed since this was not a primary purpose of this work. More data would be necessary to confirm the findings. The results were added to demonstrate that Trichomonas in general is not as fastidious an organism as was thought previously, and that the artificial culturing and the maintenance of this protozoan can still be simplified.

VI

SUMMARY AND CONCLUSIONS

The serological behavior of two Trichomonas species from the pig, as well as T. gallinarum, and T. foetus, was studied in this work.

A procedure for the living agglutination reaction of trichomonads was described and interpreted. This procedure utilized a modified antigen for the immunisation of experimental animals, and employed a different method of observing (reading) the agglutination of the living organisms under the microscope.

Antisera produced against the two major fractions of the Trichomonas cell resulted in two different types of agglutination:

1. If reacted with the antiserum produced against the water-soluble antigen fraction, all four Trichomonas species showed a typical posterior flagellar agglutination.

2. Antiserum produced against the water extracted ("insoluble") antigen fraction resulted in a typical somatic agglutination of all four Trichomonas species, and in some instances a somatic and a posterior flagellar agglutination occurred simultaneously to a lesser extent.

3. The pooling of both kinds of antisera produced both types of agglutinations, with the P.F.A. predominating in the higher dilutions and the O.A. predominating in the lower dilutions.

Cross titration studies with the homologous and the heterologous living antigens showed a strong evidence of a common group antigen in all four Trichomonas species. The isolate of T. foetus acted as a control. However, it was noted that the agglutination titers of the homologous reactions were consistently higher than the titers of the heterologous reactions. This difference in the cross-titrations was thought to be significant enough to justify a differentiation between the trichomonads used in this study, and especially, between the two species isolated from the nose and from the cecum of the pig, as reported by Sanborn (1954).

The use of ruptured trichomonads as an antigen for the immunization of experimental animals seemed to be more successful in eliciting a high antibody response, than was the application of the living or formalinized, intact trichomonad antigen reported by earlier workers. (Sanborn, 1954; Morgan, 1943, 1946; Trussell, 1946, 1947).

According to the results of the serological reactions confirmation was obtained that, like T. foetus and T. vaginalis as reported by Menolasino and Hartman (1954), also the two pig trichomonad species and T. gallinarum are highly antigenic.

Cultural studies with the media employed proved highly satisfactory, and simplified procedures could successfully be applied for all trichomonad species used in this work.

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SEROLOGICAL AND IMMUNOLOGICAL STUDIES ON SEVERAL ANIMAL TRICHOMONAS SPECIES

BY

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

year 1955

Approved by _____

With a modified procedure for the trichomonad living agglutination the serological behavior of three Trichomonas species was studied. For the production of antisera in rabbits and chicken, the trichomonads were ruptured and the antigen applied in two major fractions by intravenous injections.

Serological tests conducted with the antisera and the living antigens revealed two different types of agglutination according to the antigenic fraction of the trichomonad cell used for the immunization of the animals.

Antisera produced against the water soluble fraction of the Trichomonas cell resulted in a typical posterior flagellar agglutination, while the antisera produced against the water insoluble fractions created a typical somatic agglutination of the trichomonads, as seen under the microscope. The pooling of both kinds of antisera produced both types of agglutination simultaneously, with the P.F.A. predominating in the higher dilutions, and the O.A. predominating in the lower dilutions.

Motives for conducting these agglutination tests were: to clarify whether this method was applicable for the specific differentiation of the trichomonads, and secondly, to work towards the development of a practical testing procedure which might be useful for the diagnosis of important Trichomonas diseases.

In cross titration studies using the living antigens of Trichomonas sp. (nose of the pig), T. suis (cecum of pig), T. gallinarum, and T. foetus, a common group antigen of these species could be

noticed. It was also noticed that the titers with the homologous antisera were consistently higher than those of the heterologous reactions. This difference was thought to be significant to justify a differentiation of the trichomonads involved in this study, and particularly, of the two Trichomonas species isolated from the pig.

Contrary to reports of earlier workers (Morgan, 1943; Sanborn 1954), the observations made by Menolasino and Hartman (1954), that T. vaginalis and T. foetus are highly antigenic, could fully be confirmed also for the two Trichomonas species from the pig, and T. gallinarum.

In cultural studies with different media, it was found that simplified procedures could be applied for the trichomonads without detrimental effects. It was felt that Trichomonas in general is not as fastidious an organism as was thought previously.

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