A STUDY ON THE INHIBITION OF BACTERIA BY THE METABOLIC PRODUCTS OF TRICHOMONADS

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE
Dorothy Jean Hitchcock

1947

THESIS

This is to certify that the

thesis entitled

The Study of the Inhibition of Bacteria by the Metabolic Products of Trichomonads

presented by

Dorothy Hitchcock

has been accepted towards fulfillment
of the requirements for

M. S. degree in Parasitology

Philip A. Hawkins
Major professor

Date May 15, 1947

M-795

A STUDY ON THE INHIBITION OF BACTERIA BY THE METABOLIC PRODUCTS OF TRICHOMONADS

By

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A THESIS

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

MASTER OF SCIENCE

Department of Bacteriology and Public Health

1947

THESIS

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I. Introduction

Research has been extensively carried out to determine the inhibition of bacteria by the metabolic products of bacteria and molds. A survey of the literature revealed the production of an antibiotic by Paramecium aurelia (Sonneborn, Jacobson and Dippell, 1946; Austin, 1946). The metabolic and disintegration products of certain strains of P. aurelia when added to liquid cultures of other species of Paramecium produced toxic or lethal effects. The action of known antibiotics has been studied on protozoa. In this study the Oxford cup plate assay method was used to determine the inhibition of bacteria by the metabolic products of trichomonads.

II. Cultures

The protozoa studied consisted of bacteria-free cultures of Trichomonas foetus and Trichomonas vaginalis. The T. foetus cultures, Strains BR, O and C, were obtained from Dr. B. B. Morgan, University of Wisconsin, Madison, Wisconsin. The T. vaginalis culture, #1, was provided by Dr. G. Johnson, Ortho Research Foundation, Raritan, New Jersey.

Schneider's citrate medium (Schneider, 1942) was used for culturing and assaying the three strains of T. foetus. The medium was modified by omitting the brom cresol purple. Each tube of medium, consisting of 5 ml. of egg-blood mixture slanted, and 12 ml. of overlay containing 6.5% bovine serum and egg shells, was autoclaved at 121°C, 15# for 30 min. The uninoculated medium was stored in the refrigerator and incubated 3 days at 37°C for sterility.

The T. foetus cultures were subcultured once a week by transferring approximately 1 ml. of overlay with a sterile opsonic pipette to a fresh tube of Schneider's citrate medium. The subcultures were incubated the first two days at 37°C, and the remainder of the time until assayed at 22°C.

The T. vaginalis culture, #1, was maintained and assayed in cysteine-peptone-liver-maltose medium (C. P. L. M.) (Johnson and Trussel, 1943) modified by omitting the methylene blue and agar. The base medium was tubed in 9 ml. amounts and autoclaved at 121°C, 15# for 30 min. After the addition of 1 ml. of sterile horse serum to each tube, the medium was incubated 4 days at 37°C and examined grossly for contamination.

The T. vaginalis cultures were subcultured every 2 to 3 days by transferring approximately 0.5 ml. of the culture with a sterile opsonic pipette

to a fresh tube of C. P. L. M. medium. The subcultures were incubated at 37°C until assayed.

The bacterial cultures were kept in stock on slants of the base assay agar (Schmidt and Moyer, 1944) and subcultured once a month. The seed inoculum bacterial cultures were subcultured daily from broth to broth (Schmidt and Moyer, 1944) and incubated at 37°C. Every two weeks the broth bacterial cultures were started from stock cultures. The bacteria chosen for this study, tables 1 and 13, were limited to those that would grow satisfactorily on the base agar, give even turbidity in the broth and were not too prolific growers in the 22-hr. assay incubation period.

III. Methods

The Oxford cup plate assay method used for T. foetus is described, followed by the modifications used for T. vaginalis.

The Petri plates of 20 ml. of base agar (Schmidt and Moyer, 1944) were poured and left at room temperature 16-18 hours before seeding. The bacterial inoculum, consisting of 3 ml. of base agar seeded with a 1% 24-hr. broth culture (Schmidt and Moyer, 1944) of a bacterium, was evenly distributed over the 20 ml. of base agar. When the seed inoculum was firm, five sterile porcelain Oxford cups, warmed on a hot plate, were evenly arranged on each assay plate. The T. foetus cultures to be tested for bacterial inhibition were examined microscopically for sufficient growth of the trichomonads, tested for bacterial sterility in broth, pH determined with nitrazine paper, and centrifuged at 1150 r.p.m. for 10 minutes. The supernatant fluid was decanted, thus removing the trichomonads from the protozoan culture material to be assayed. Four drops of the supernatant of the centrifuged protozoal culture material were placed in three of the cups by means of a sterile 1 ml. pipette. Four drops of uninoculated control medium were added to the control cup. The control was treated in the same manner as the protozoal cultures, except the pH was adjusted to 5.0-5.5 with HCl. This pH adjustment was necessary as the T. foetus after incubation lowers the pH from 7.2-7.4 to 5.0-5.5. One cup, to which no fluid was added, served as a heat control. The assay plates were covered with sterile porcelain lids and incubated 22 hrs. at 37°C. The zones of inhibition were observed by reading over a substage microscope lamp and measured in millimeters with a ruler. One of the zones of inhibition from each plate was touched on the surface with a

sterile wire and seeded to broth to determine if the inhibitory substance was bacteriøcidal or bacteriostatic.

Each assay on each tube of protozoal culture against one bacterium consisted of three plates with five Oxford cups per plate; three of these for the centrifuged protozoal culture material, one for the uninoculated, adjusted medium control and one for a control on the heat of the cup. A complete assay consisted of three protozoal cultures of the same strain and same age against three bacteria, requiring a total of 27 plates. At least two complete assays were done with 7, 10 and 12 day T. foetus cultures, Strains BR, O and C, against the bacteria listed in table 1.

The above procedure was modified with T. vaginalis by allowing the base agar plates to stand 12-14 hrs. at room temperature before seeding, centrifuging at 1150 r.p.m. for 15 min. and assaying 2 to 3 day cultures against the bacteria listed in table 13. Three complete assays were done with the T. vaginalis cultures against each bacterium.

Assays were also done on T. foetus, Strain BR, 10 day cultures grown in Schneider's citrate medium without the citrate and brom cresol purple. T. foetus, Strain BR, was maintained 8 weeks on this modified Schneider's citrate medium. Weekly transfers were made and subcultures incubated the first two days at 37°C and remainder of time until assayed at 22°C.

Quantitative citric acid determinations (Pucher, Vickey and Leavenworth, 1934) were done on pooled T. foetus, Strain BR, 10 day cultures showing inhibition against Salmonella pullorum by the above-described assay method. An equal number of pooled tubes of uninoculated, unadjusted Schneider's citrate medium served as controls. The quantitative citric acid determinations were done on known volumes of the centrifuged trichomonad cultures and controls as described in the cited reference except

the filtration through Gooch crucibles was omitted and extractions were done in a Soxhlet apparatus using Whatman fat extraction thimbles, 33x94 mm. Two determinations were done on each sample of pooled protozoal culture material and on each pooled control.

IV. Discussion

Ten-day cultures of T. foetus, Strains BR, O and C, produced inhibition of S. pullorum. Seven-day cultures of Strain BR showed inhibition of Corynebacterium renale and Salmonella schottmuelleri. The other bacteria listed in table 1 were not inhibited by 7, 10 or 12-day cultures of Strains BR, O or C, as determined by this assay method.

Table 2 shows the percentage of T. foetus assay cups showing no inhibition, fewer colonies or definite inhibition against S. pullorum. With 7 and 10-day cultures of Strain BR, 42% of the cups showed inhibition. The maximum percentage of cups showing inhibition with Strain BR were 12-day cultures and with Strain O, 10-day cultures. With Strain C the older the culture the higher the percentage of inhibition. Attention should be called to the fact that the least number of cups (75) were used with 12-day cultures of Strain C which had the highest percentage of inhibition.

Figure 1 was prepared for clarification of the data pertaining to the percentage of cups showing inhibition in table 2. T. foetus, Strains O and C, 7-day cultures, showed no inhibition against Corynebacterium renale and Salmonella schottmuelleri. Strain BR, 7-day cultures, produced 10% inhibition of C. renale, table 3, and 9% inhibition of S. schottmuelleri, table 4. There is need for further study before evaluating this data.

With all strains of T. foetus the largest zones of inhibition of S. pullorum, table 5, were with the oldest cultures, i.e., 12 day. Strain BR produced the maximum zones of inhibition of S. pullorum of the three strains for 7, 10 and 12-day cultures.

Of the 95 cups showing inhibition of S. pullorum, table 6, 50% were

shown to be bacteriostatic and 50% bacteri$cidal when seeded in broth.

For T. foetus, Strain BR, assayed on Schneider's citrate medium, modified by omitting the sodium citrate, the percentage of cups showing inhibition, table 7, increased with the age of the protozoal culture. This data compares favorably with the data in table 2 grown in Schneider's citrate medium containing the citrate.

Of all the T. foetus cultures, grown in Schneider's citrate medium, inhibitory and non-inhibitory for S. pullorum, the maximum percentage of them showed a pH of 5.5, table 8, with the exception of Strain 0, 12-day cultures, which showed the highest percentage at a pH of 6.0.

Table 8 was separated into tables 9 and 10, grouping the pH of cultures showing inhibition in table 9 and those non-inhibitory in table 10. The maximum percentage of the cultures, showing inhibition of S. pullorum, had a pH of 5.5, table 9. The two exceptions were 7 and 12-day cultures of Strain 0, which showed the highest percentage of cultures with a pH of 6.0.

The maximum percentage of T. foetus cultures, non-inhibitory for S. pullorum, had a pH of 5.5, with the exception of Strain 0, 10 and 12-day cultures, table 10. The variation in pH of inhibitory and non-inhibitory cultures is not of great enough significance to account for the inhibition of S. pullorum.

The pH of T. foetus, Strain BR, grown in Schneider's citrate medium modified by omitting the sodium citrate, table 11, closely similates the pH of the same strain and same age found in table 8.

On the possibility that the inhibition of S. pullorum was due to the sensitivity of this bacterium to citric acid, quantitative citric acid determinations, table 12, were done to determine the amount of citrate

synthesized or utilized by T. foetus, Strain BR, 10-day cultures. The amount of variation in mgm. per ml. of citrate is negligible and within the accuracy of the method.

T. vaginalis, #1, 2 and 3-day cultures, grown in C. P. L. M. medium produced no inhibition of the bacteria listed in table 13 by the described assay method. Streptococcus agalactiae and Eberthella typhosa were unsuitable for assay by this method as a white precipitate formed around each assay cup and the control cup.

Some substance or substances in cultures of T. foetus produced inhibition of S. pullorum, C. renale and S. schottmuelleri. There was no attempt made to determine the chemical nature of this material or to concentrate it. However, there is substantial evidence which indicates that it is not alone associated with the pH of the culture. The substance tends to increase in the medium on cultivation whereas the pH of the medium is relatively stable at a pH of about 5.5 for the 7, 10 and 12-day cultures. Further there is no relation between the pH of the culture and the production or non-production of the inhibitory substance. The mode of action of the material is not known since in different tests it has been bacteriostatic and bactericidal. This would probably indicate a difference in concentration of the inhibitory substance.

V. Results

Table 1

Bacterial Inhibition Produced by *Trichomonas foetus* Schneider's Citrate Medium

Bacteria	7-day cultures		
Strains	BR	O	C
Salmonella pullorum	+	+	+
S. typhimurium	-	-	-
S. schottmuelleri	+	-	-
Corynebacterium renale	+	-	-
Shigella gallinarum (M.S.C.)	-	-	-

	10-day cultures		
Strains	BR	O	C
Salmonella pullorum	+	+	+
S. goettingen	-	-	-
S. gallinarum (U. of Kentucky)	-	-	-
S. cholerasuis	-	-	-
Staphylococcus aureus 209	-	-	-
Aerobacter aerogenes	-	-	-

	12-day cultures		
Strains	BR	O	C
Salmonella pullorum	+	+	+
S. paratyphi	-	-	-
S. enteriditis	-	-	-

\+ Inhibition

\- No inhibition

Table 2

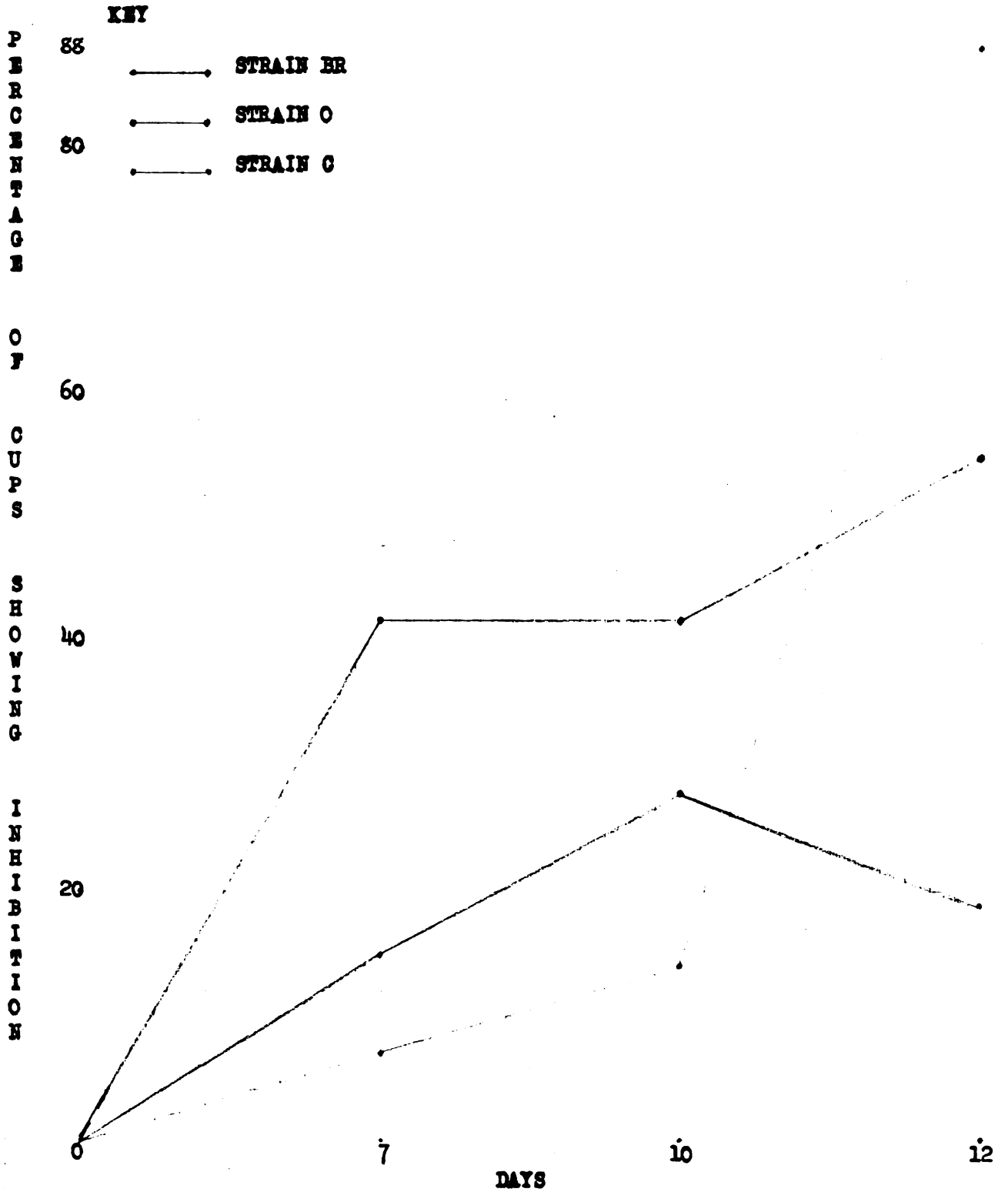
Percentage of T. foetus assay cups showing inhibition of Salmonella pullorum. Schneider's citrate medium

Age of Cultures	7 days			10 days			12 days		
Strains	BR	O	C	BR	O	C	BR	O	C
No inhibition	50%	67%	85%	41%	52%	75%	38%	69%	3%
Fewer colonies	8	18	8	17	20	11	7	12	9
Inhibition	42	15	7	42	28	14	55	19	88
No. of cups	299	162	182	299	120	162	170	81	75

INHIBITION OF *SALMONELLA PULLORUM*

BY *TRICHOMONAS FOETUS*

SCHNEIDER'S CITRATE MEDIUM



AGE OF *T. FOETUS* CULTURES

Table 3

Percentage of T. foetus assay cups showing inhibition of Corynebacterium renale. Schneider's citrate medium

Age of Cultures	7 days		
Strains	BR	O	C
No inhibition	80%	0	0
Fewer colonies	10	0	0
Inhibition	10	0	0
No. of cups	150	48	78

Table 4

Percentage of T. foetus assay cups showing inhibition of Salmonella schottmuelleri. Schneider's citrate medium

Age of Cultures	7 days		
Strains	BR	O	C
No inhibition	67%	0	0
Fewer colonies	24	0	0
Inhibition	9	0	0
No. of cups	54	75	102

Table 5

Average zones of inhibition
of *S. pullorum* by *T. foetus*
Schneider's citrate medium

Age of Cultures	7 days	10 days	12 days
Strains			
BR	6.2 mm.	6.0 mm.	7.9 mm.
O	4.1	4.9	6.9
C	5.0	4.4	5.8

Of *Corynebacterium renale* by *T. foetus*
Schneider's citrate medium

Age of Cultures	7 days
Strains	
BR	4.3 mm.
O	0
C	0

Of *Salmonella schottmuelleri* by *T. foetus*
Schneider's citrate medium

Age of Cultures	7 days
Strains	
BR	2.5 mm.
O	0
C	0

Table 6

Inhibition of S. pullorum by T. foetus
Bactericidal or Bacteriostatic Action

Age of Cultures	7 days			10 days			12 days			Total
Strains	BR	O	C	BR	O	C	BR	O	C	Cups
Bactericidal	7	2	2	9	8	5	2	4	9	48
Bacteriostatic	14	0	0	9	3	5	0	3	13	47
Total cups	21	2	2	18	11	10	2	7	21	95

Table 7

Percentage of T. foetus assay cups showing inhibition of Salmonella pullorum Schneider's citrate medium without the citrate

Strain BR

Age of Culture	7 days	10 days	12 days
No inhibition	50.0%	50.0%	11.1%
Fewer colonies	33.3	13.7	37.0
Inhibition	16.6	36.2	51.4
No. of cups	54	204	27

Table 8

pH of *Trichomonas foetus* cultures
Inhibitory and non-inhibitory. Schneider's citrate medium

Age of Cultures	7 days			10 days			12 days		
Strains	BR	O	C	BR	O	C	BR	O	C
pH 5.0	2.6%	-	-	-	-	-	8.7%	-	-
5.1	2.6	-	-	-	-	-	-	-	-
5.2	13.1	11.1%	4.7%	23.3%	6.6%	5.5%	13.1	-	-
5.3	5.2	-	-	-	-	-	4.3	-	-
5.4	-	-	-	-	-	-	4.3	-	-
5.5	50.0	50.0	52.3	52.4	53.3	44.4	52.1	25.0%	66.6%
5.6	-	-	-	-	-	-	4.3	-	-
5.7	15.7	5.5	19.0	14.2	26.6	16.6	4.3	25.0	11.1
5.8	-	-	-	-	-	-	-	-	-
5.9	-	-	-	-	-	-	-	-	-
6.0	5.2	33.3	14.2	9.5	13.3	16.6	8.2	50.0	22.2
6.1	-	-	-	-	-	-	-	-	-
6.2	-	-	-	-	-	-	-	-	-
6.3	5.2	-	-	-	-	-	-	-	-
6.4	-	-	-	-	-	-	-	-	-
6.5	-	-	9.5	-	-	5.5	-	-	-
7.0	-	-	-	-	-	11.1	-	-	-
Total cultures	36	18	21	21	15	18	23	8	9

- No data

Table 9

pH of T. foetus cultures
Inhibition of S. pullorum
Schneider's citrate medium

Age of Cultures	7 days			10 days			12 days		
Strains	BR	O	C	BR	O	C	BR	O	C
pH 5.0	5.2%	-	-	-	-	-	15.3%	-	-
5.1	5.2	-	-	-	-	-	-	-	-
5.2	26.3	14.2%	-	41.6%	11.1%	20.0%	15.3	-	-
5.3	10.5	-	-	-	-	-	7.6	-	-
5.4	-	-	-	-	-	-	7.6	-	-
5.5	47.3	28.5	40.0%	50.0	77.7	80.0	46.1	25.0%	66.6%
5.6	-	-	-	-	-	-	7.6	-	-
5.7	-	-	40.0	-	11.1	-	-	25.0	11.1
5.8	-	-	-	-	-	-	-	-	-
5.9	-	-	-	-	-	-	-	-	-
6.0	5.2	57.1	20.0	0.8	-	-	-	50.0	22.2
Total inhibitory cultures	19	7	5	12	9	5	13	4	9

- No data

Table 10

pH of *T. foetus* cultures
Non-inhibitory for *S. pullorum*
Schneider's citrate medium

Age of Cultures	7 days			10 days			12 days		
Strains	BR	O	C	BR	O	C	BR	O	C
pH 5.0	-	-	-	-	-	-	-	-	-
5.1	-	-	-	-	-	-	-	-	-
5.2	-	9.0%	6.2%	-	-	-	10.0%	-	-
5.3	-	-	-	-	-	-	-	-	-
5.4	-	-	-	-	-	-	-	-	-
5.5	47.0%	63.6	56.2	55.5%	16.6%	30.7%	60.0	25.0%	-
5.6	-	-	-	-	-	-	-	-	-
5.7	35.2	9.0	12.5	33.3	50.0	23.0	10.0	25.0	-
5.8	-	-	-	-	-	-	-	-	-
5.9	-	-	-	-	-	-	-	-	-
6.0	5.8	18.1	12.5	11.1	33.3	23.0	20.0	50.0	-
6.1	-	-	-	-	-	-	-	-	-
6.2	-	-	-	-	-	-	-	-	-
6.3	11.6	-	-	-	-	-	-	-	-
6.4	-	-	-	-	-	-	-	-	-
6.5	-	-	12.5	-	-	7.6	-	-	-
7.0	-	-	-	-	-	15.3	-	-	-
Total non-inhibitory cultures	17	11	16	9	6	13	10	4	0

- No data

Table 11

pH of Trichomonas foetus cultures, Strain BR
Inhibitory and non-inhibitory
Schneider's citrate medium without the citrate

Age of Cultures	7 days	10 days	12 days
pH 5.0	-	19%	-
5.1	-	-	-
5.2	50%	23.8	33.3%
5.3	-	-	-
5.4	-	-	-
5.5	33.3	47.6	66.6
5.6	-	-	-
5.7	16.1	4.7	-
5.8	-	-	-
5.9	-	-	-
6.0	-	4.7	-
Total cultures	6	21	3

- No data

Table 12

Quantitative citric acid determinations
Trichomonas foetus, Strain BR, 10-day cultures
Inhibitory for S. pullorum. Schneider's citrate medium

No.	Media Lot No.	mgm./ml. Culture Sample 1	mgm./ml. Culture Sample 2	Average mgm./ml. of Sample	mgm./ml. Control Sample A	mgm./ml. Control Sample B	Average mgm./ml. Controls	mgm./ml. Variation
1	11	3.63	3.58	3.60	3.58		3.67	dec. 0.07
2	11	3.81	3.58	3.69		3.76		inc. 0.02
3	11	3.54	3.31	3.40	3.72		3.61	dec. 0.22
4	11	3.50	3.72	3.61		3.50		No change
5	11	3.88	4.08	3.98	3.74		3.69	inc. 0.29
6	11	3.54	3.81	3.67		3.63		inc. 0.04
				Average variation			inc. 0.01 mgm./ml.	

Table 13

Bacterial inhibition produced by Trichomonas vaginalis, #1
G. P. L. M. medium

2-day cultures

Bacteria	
Salmonella schottmuelleri	-
S. typhimurium	-
S. pullorum	-
S. choleraesuis	-
S. gallinarum (U. of Kentucky)	-
S. paratyphi	-
S. enteriditis	-
Aerobacter aerogenes	-
Pseudomonas aeruginosa	-
Escherichia coli	-

3-day cultures

Staphylococcus aureus 209	-
Staph. aureus H	-
Shigella gallinarum (M. S. C.)	-
S. sonnei	-
S. paradysenteriae Boyd 88	-
S. paradysenteriae Flexner "Y"	-

- No inhibition

VI. Conclusions

1. By the described Oxford cup plate assay method *T. foetus*, Strains BR, 0 and C, 7, 10 and 12-day cultures, produced inhibition of *S. pullorum*.
2. *T. foetus*, Strain BR, 7-day cultures inhibited *C. renale* and *S. schottmuelleri*. Further study is needed as a limited number of cultures were assayed.
3. Sodium citrate is not necessary in the Schneider's citrate medium for the growth or for production of inhibition of *S. pullorum* by *T. foetus*, Strain BR.
4. The pH of the *T. foetus* cultures was not responsible for the inhibition of *S. pullorum* in Schneider's citrate medium or in the medium modified by omitting the sodium citrate.
5. Citrate is not synthesized or utilized by *T. foetus*, Strain BR, 10-day cultures.
6. *T. vaginalis*, #1, 2 and 3-day cultures did not produce inhibition of bacteria by the described assay method.

VII. Acknowledgment

The writer wishes to take this opportunity to express her appreciation to Drs. Philip Hawkins, Garth Johnson, Banner Bill Morgan, Nicholas Pattu, members of the Department of Bacteriology and Michigan Department of Health for their assistance in this study.

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