THE EFFECTS OF BACTERIA ON THE GROWTH OF TRICHOMONAS FOETUS (PROTOZOA)

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Very little work has been reported concerning the effects of bacteria on the growth in vitro of Trichomonas foetus. Numerous reports in the literature indicate that many trichomonad infections in cattle are accompanied by a varied bacterial flora. The influence these organisms may have on the ultimate establishment of bovine trichomoniasis is not known. The purpose of this paper is to present research concerning the effect of certain bacteria upon the growth of T. foetus in vitro.

Reidmüller (1928) isolated Brucella abortus, gram-negative rods, diplococci, and streptococci from aborted fetuses in which trichomonads were found. Abelein (1929), Weidenauer (1930), and Kohl (1933) found diplococci, streptococci, staphylococci, and diphtheroids in trichomonad pyometra fluid. Hahn (1935) and Wohlfarth (1937) identified Corynebacterium pyogenes in cases of trichomonad pyometra. Smythe (1943) and Murray (1943) also reported the coexistence of C. pyogenes and T. foetus in cattle.

Kerr (1942) pointed out that lesions in cattle infected with T. foetus may have been due to associated or concurrent secondary bacterial infections by such organisms as diphtheroids or streptococci. Kerr (1943) also recognized the presence of bacterial infection in an abscessed condition of the epididymis of a bull that had been inoculated with a diphtheroid-contaminated culture of T. foetus. Karlson and Boyd (1941) found T. foetus in the seminal vesicle of a bull with bilateral orchitis and vesiculitis; a pure culture of C. pyogenes was present in both seminal vesicles and the left epididymis. Patrizi (1940) reported on a case of genital tuberculosis accompanied by trichomoniasis in a bull.

Abelein (1929), Reidmüller (1928), and Gehring and Murray (1933) were unsuccessful in cultivating T. foetus free from bacteria. Witte (1933) was the first to cultivate a pure strain of this flagellate. He inoculated bacteria-free trichomonad pyometra exudate into serum broth cultures. Glaser and Coria (1935) described the “V” tube migration technique and isolated in pure culture the first American strain of T. foetus. Since their report, many methods of isolation have been suggested.

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It has been known for some time that *T. foetus* will not grow in vitro in the presence of certain contaminating bacteria. Morgan (1942) first demonstrated that an atypical strain of *Corynebacterium renalis* grows in close association with *T. foetus* since the flagellates multiplied more rapidly in its presence than in bacteria-free cultures. He believed that this acceleration may be caused by a change in the medium due to hydrolysis of proteins and carbohydrates by the diphtheroid, but the nature of this action is not known.

Plastridge (1943) was unable to grow *T. foetus* in the presence of staphylococci and coliform organisms. Zeetti (1940) found trichomonads could not grow with various species of micrococci. Williams and Plastridge (1946) found that *T. foetus* was completely destroyed in less than 24 hours by *Streptococcus viridans*, *Streptococcus faecalis*, *Streptococcus lactis*, *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus vulgaris*, and in 48 hours by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Moderate growth occurred in the presence of *Micrococcus tetragenus*, *Sarcina lutea*, and *Bacillus subtilis*. These investigators and Morgan (1946) were successful in freeing trichomonad cultures of most contaminating bacteria by the use of penicillin and streptomycin.

**MATERIALS AND METHODS**

*Culture medium.* The culture medium employed was a modification of Schneider's (1942) citrate medium. This medium has proved to be very satisfactory for the growth of *T. foetus* and all of the bacteria studied in this work.

Schneider's modified medium was prepared in 2 portions: slant and supernatant. A citrate solution was first made and is of the following composition: sodium chloride, 5 g; magnesium sulfate, 0.2 g; ammonium phosphate (monobasic), 1 g; potassium phosphate (dibasic), 1 g; sodium citrate, 2 g; glucose, 10 g; and distilled water to 1,000 ml.

The slant portion of the medium was then prepared. Six fresh eggs were thoroughly mixed with 60 ml of citrated bovine blood and 75 ml of the citrate solution by agitation in a flask containing glass beads. This suspension was then filtered through cheese cloth, and 2.5-ml portions were tubed in 16-mm test tubes. The tubes were slanted and insipissated in an autoclave for 5 to 10 minutes. Air was not evacuated from the chamber during inspissation. When carefully done, smooth glistening slants resulted.

To prepare the liquid supernatant, 500 ml of the citrate solution were added to 50 ml of bovine serum, 8 ml of hemat solution (50 mg in 20 ml of distilled water), and 0.5 ml of a 1.6 per cent aqueous solution of bromcresol purple. This solution was then filtered through a double thickness of coarse filter paper and 5 ml were added to each slanted tube. The tubes were plugged with cotton, autoclaved for 30 minutes at 15 pounds' pressure, and stored in the refrigerator until used. The final pH of the medium was approximately 7.0 to 7.2.

*Organisms employed.* The trichomonad employed in these studies was strain "BR", which was routinely maintained in the laboratory. It was originally isolated by Morgan and Wisnicky (1942) from a cow suffering from a trichomonad pyometra and has since been kept in pure culture on the modified Schneider's medium.
Cultures of Corynebacterium hofmannii, Corynebacterium xerosis, Corynebacterium diphtheriae, Streptococcus hemolyticus, Streptococcus salivarius, Vibrio comma, Neisseria catarrhalis, Staphylococcus aureus, Staphylococcus albus, Shigella dysenteriae, and Shigella paradysenteriae were kindly supplied by the Department of Medical Bacteriology, Medical School, University of Wisconsin. Corynebacterium renale (atypical; this diphtheroid would not ferment glucose), C. pyogenes, Streptococcus bovis (5 strains), Streptococcus faecalis (2 strains), Acrobacter aerogenes, and Escherichia coli, all recently isolated from bovine pyometras, were obtained from the Department of Veterinary Science, College of Agriculture, University of Wisconsin. Brucella abortus (strain 19) was also supplied by this department.

Through the kindness of the Department of Agricultural Bacteriology, College of Agriculture, University of Wisconsin, cultures of Pseudomonas aeruginosa, Bacillus subtilis, and Corynebacterium equi were obtained.

An unidentified Sarcina was isolated by the writers from a sheath swab of an apparently normal bull used in an artificial insemination ring. This organism was nonmotile, gram-negative, did not liquefy gelatin or Loeffler’s blood serum, fermented glucose, and formed a deep yellow pigment. Corynebacteria cultures 714, 154SH, 49, and RIDG5, and strains 161SH and 152SH of Corynebacterium renale were isolated by Morgan et al. (1946) from sheath swabs of apparently normal breeding bulls. Cultures B-1, B-2, and B-3 of diphtheroids were recovered by Morgan et al. (1946) from the seminal vesicle of a bull infected with T. foetus.

Determination of growth rates. The counting of T. foetus in the various cultures inoculated was accomplished with a Levy-Hausser hemocytometer counting chamber. The culture tubes were carefully rotated and samples withdrawn aseptically with a 6-mm wire loop.

All cultures in this work were incubated at 37 C. An inoculum of approximately 50,000 active flagellates per tube was standard throughout this study. Contamination was guarded against constantly and tested for periodically by the examination of films stained with Loeffler’s methylene blue. Contaminated cultures were discarded and replaced by others in order to keep the series constant for direct comparisons. Tubes of freshly prepared uninoculated media were incubated for 24 hours at 37 C and examined for bacterial growth as an added precaution against contamination.

Before study of the effect of various bacteria, bacterial filtrates, and heat-killed bacterial cultures on the growth of this trichomonad, the normal growth rate of T. foetus employing a constant inoculum in a constant quantity of medium was determined. A total of 178 replicate cultures were counted to establish the normal growth curve of T. foetus.

In the experiments utilizing live bacteria, 5 tubes of Schneider’s medium were inoculated simultaneously with trichomonads and a loop of a 24-hour bacterial culture was grown in the same medium. Brucella abortus (strain 19) and C. renale (strain 152SH) were incubated for 48 hours before inoculation since 24 hours was not sufficient to permit adequate growth.

Studies with killed bacteria were made on 24-hour cultures which were grown
in Schneider's medium and heat-killed in a 60 C water bath for 1 hour. After cooling, 5 tubes of the heat-killed cultures were inoculated with T. foetus for each species of bacteria tested. The effect of killed bacteria on the growth of this trichomonad was determined with the following bacteria: C. renale (atypical), C. pyogenes, C. hofmannii, C. parvum, C. diphtheriae, Streptococcus hemolyticus, S. salivarius, S. bovis (5 strains), S. faecalis (2 strains), V. comma, Neisseria catarrhalis, Staphylococcus aureus, S. albus, Shigella dysenteriae, S. paradysenteriae, A. aerogenes, and E. coli.

Seitz filtrates of 24-hour bacterial cultures were prepared from the liquid portion of Schneider's medium. These filtrates were aseptically pipetted into tubes containing only the inspissated blood-egg slant and inoculated with T. foetus. Five such tubes were inoculated for each species of bacterium studied. Filtrates were made from the following bacteria: C. renale (atypical), C. hofmannii, C. parvum, C. diphtheriae, S. bovis (strains 2 and 4), S. faecalis (strain 1), S. hemolyticus, S. salivarius, Shigella dysenteriae, Shigella paradysenteriae, Neisseria catarrhalis, and E. coli.

The change in pH of the medium in which the control cultures were grown was determined with quinhydrone (Leeds and Northrup) and glass electrode (Coleman) potentiometers. Cultures other than the controls were not checked potentiometrically for pH changes. A qualitative pH trend was noted by observation of a color change in the indicator bromocresol purple.

RESULTS

Control cultures. The growth curve derived from multiplication of T. foetus in pure culture employing an inoculum of 50,000 organisms in 5 ml of medium was obtained from 2,487 counts on 178 cultures and can be noted in figures 1 to 6. An initial stationary phase and a lag phase were not evident. Apparently, the flagellate began logarithmic growth at the time of inoculation and continued in this phase for approximately 72 hours of incubation. The trichomonads multiplied at a decreased rate from 72 to 92 hours, and from 92 to 108 hours the flagellates had reached their maximum stationary growth phase. The peak of growth was attained in 100 hours, at which time the direct count was approximately 3.5 million organisms per ml. The period from 108 to 144 hours might be considered the phase of accelerated death, and the period from 144 to 216 hours the logarithmic death phase. After 216 hours, the numbers of trichomonads decreased very rapidly until none could be counted after 240 hours of incubation.

The initial pH of 7.2 was gradually reduced to a final pH of 5.18. The pH change was most rapid from 36 hours to 144 hours. After 144 hours the pH decreased from 5.4 to 5.18.

Corynebacteria. Figure 1 shows the effect of six species of corynebacteria on the development of T. foetus in vitro. Nine cultures of diphtheroids isolated from the genital tract of bulls were tested for their effect on the growth rate of this trichomonad, and the results are represented in figures 2 and 3.

The most striking point concerning figure 1 is the extreme prolongation of
life afforded *T. foetus* when grown in association with *C. equi*. This necessitated the use of a different horizontal scale from that used to construct the other five graphs. In the presence of *C. equi* the trichomonads reached their
peak of growth in 100 hours, as did the control cultures, but this peak was approximately 14 million organisms per ml as compared to the peak of 3.5 million reached by the control. One of the former replicate cultures attained a peak of approximately 18 million per ml. The longevity of *T. foetus* was extended from 240 to 528 hours by *C. equi*. With *C. renale*, *C. hofmannii*, and *C. xerosis*, growth of the trichomonads was initially accelerated for but a brief time. The peak of growth of trichomonads was reached in 88 hours with *C. renale* and *C. hofmannii* and in 62 hours with *C. xerosis*; death occurred in 202, 160, and 136 hours, respectively. *C. pyogenes* and *C. diphtheriae* were decidedly inhibitive of trichomonad growth. Both moved the peak of trichomonad activity to the left at 48 hours, and the death of the trichomonads occurred in 110 hours with *C. pyogenes* and in 88 hours with *C. diphtheriae*. With the exception of *C. equi*, none of the species of *Corynebacterium* in figure 1 allowed *T. foetus* to attain a peak of growth as high as the control cultures.

In figures 2 and 3, diphtheroids are represented which were recovered in a previous study (Morgan *et al.*, 1946) from sheath swabs of clinically normal bulls with the exception of cultures B-1, B-2, and B-3, which were isolated from the seminal vesicle of a trichomonad-infected bull. Only two cultures, 152SH and 161SH, were identified; these were strains of *C. renale*. All of the diphtheroids accelerated multiplication of *T. foetus*, whereas 3 of them, B-1, B-2, and B-3, did not permit so high a peak of growth as was reached by the control. Most of the diphtheroids shifted the peak of trichomonad growth from 100 to 72 hours. One of the diphtheroids, B-1, was interesting in that it maintained the
maximum stationary phase of trichomonad growth from 72 to 132 hours as compared to the range of 92 to 108 hours maintained by the control. Death of the flagellates occurred in 192, 216, and 264 hours with cultures 152SH, 49, and B-1, respectively. The other diphtheroids approximated the control in that activity of *T. foetus* subsided in about 240 hours.

**Streptococci.** Figure 4 indicates that in general the 10 streptococci studied are inhibitory to the multiplication of *T. foetus*. Only *S. bovis* (strain 5) gave any acceleration to the growth of the flagellates beyond that of the control growth curve, but this was short-lived with death resulting in 88 hours. *S. pyogenes* permitted longer survival of *T. foetus* than did other streptococci, but there was no acceler-

![Figure 4. Growth of Trichomonas foetus with Various Species of Streptococci](image)

Fig. 4. Growth of Trichomonas foetus with Various Species of Streptococci

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nosa, Brucella abortus (strain 19), and Sarcina sp. was accelerated. N. catarrhalis slightly inhibited T. foetus. The peak of growth reached by T. foetus when growing with these cultures was equal to or, in most cases, greater than that of the control. Death of T. foetus occurred later (260 hours) than with the control
only with Sarcina sp. Williams and Plasteridge (1946) obtained somewhat different results with P. aeruginosa. They found that with a small inoculum T. foetus was killed within 48 hours when cultured with this bacterium.

The enteric group of bacteria, E. coli, A. aerogenes, Shigella dysenteriae, and Shigella paradysenteriae, and 2 species of staphylococci (S. aureus and S. albus) greatly inhibited the growth of T. foetus. The longevity of the trichomonads did not extend beyond 88 hours with any of these bacteria. T. foetus lived up to 48 hours with E. coli and A. aerogenes, whereas Williams and Plasteridge (1946), using a smaller inoculum, found these bacteria to kill trichomonads in less than 24 hours. These investigators also found S. aureus to kill trichomonads in less than 48 hours as compared to the 88-hour limit shown in these results.

Brucella abortus (strain 19) stimulated the growth of the trichomonads to as great a degree as any of the bacteria studied. When cultured in the presence of this bacterium, T. foetus reached a peak of over 11 million organisms per ml at 72 hours. However, the numbers of trichomonads declined rapidly thereafter until none could be found at 192 hours.

When grown with V. comma, the trichomonads showed a very slight initial growth acceleration over that of the control, but after 48 hours' incubation the growth curve stayed below the control, and the death of T. foetus occurred within 180 hours.

Growth of T. foetus with filtrates of bacterial cultures and heat-killed bacterial cultures. The development of T. foetus in association with various bacterial filtrates was essentially the same as that found with living bacteria. Trichomonad development in media containing heat-killed bacteria paralleled that already discussed for living bacteria and bacterial filtrates, with the exception of 3 species. With heat-killed C. xerosis cultures, growth was arrested some 20 hours later than in live cultures of this corynebacterium. Again, the maximum number of organisms did not reach that found in the bacteria-free control tubes. A slight prolongation of growth of T. foetus occurred in heat-killed cultures of S. pyogenes and V. comma.

DISCUSSION

The factors resulting in acceleration and inhibition of the growth of T. foetus with various bacterial cultures, filtrates, and heat-killed suspensions are not completely understood. A change in the hydrogen ion concentration of the medium may play a part, and, since the medium contains glucose, a higher acidity in general results from those bacteria capable of fermenting this monosaccharide than from those that do not. All of the bacteria that produced an inhibition of growth of the flagellate fermented glucose. However, some of the bacteria that accelerated the development of T. foetus also fermented glucose. T. foetus is capable of withstanding an extremely wide acid pH range (Morgan and Campbell, 1945), but, with certain rapidly growing bacteria, it may not be able to adjust itself to sudden pH reductions.

The exotoxin-producing bacteria were inhibitory to T. foetus without exception. The bacteria that killed trichomonads the quickest were not necessarily toxin
producers since *A. aerogenes* and *E. coli* were more inhibitory than any bacterium studied with the exception of *S. bovis* (strain 3).

Since bacterial filtrates were comparable to living bacterial cultures in action upon *T. foetus*, it might be expected that the heat-killed toxin-producing species would, as a result of inactivating thermolabile toxins, affect the growth of the trichomonads in a different manner. However, there is some doubt that the heat treatment of 1 hour at 60 C was sufficient to destroy these toxins since, in most cases, there was such slight difference between the growth of *T. foetus* with living and heat-killed cultures of toxin-producing species. There appears to be little evidence from these results concerning the role of toxin production and its effect on the viability of *T. foetus*.

Some bacteria perhaps release valuable food products that would be unavailable to the trichomonads in pure culture. This may be the reason for the extreme prolongation of life of *T. foetus* by *C. equi* (figure 1). The unusual action by *C. equi* may be attributed to the release of growth factors contained in the blood-egg portion of the modified Schneider's medium used in these studies. Unlike any of the other bacteria, *C. equi* caused an almost complete disintegration of the slant.

The diphtheroids (figures 2 and 3) were more consistently accelerative in their action upon *T. foetus* than any other group represented in this research. These organisms are common inhabitants of the uro-genital tract of cattle and are found frequently in close association with *T. foetus in vivo* (Morgan et al., 1946). These organisms may play a part in the establishment of bovine genital trichomoniasis.

It seems that the action of various bacteria upon the growth of *T. foetus* involves three factors, namely, change in the hydrogen ion concentration, toxin production, and enzymatic release of growth substances in the medium which would be otherwise unavailable to the trichomonads.

**SUMMARY**

Certain bacteria have been found to accelerate and others to inhibit the growth *in vitro* of *Trichomonas foetus* as determined by direct microscopic counts of the trichomonads with a hemocytometer.

Bacteria that definitely inhibited the growth of *T. foetus* were *Corynebacterium diphtheriae*, *Corynebacterium pyogenes*, *Corynebacterium xerosis*, *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus bovis* (five strains), *Streptococcus faecalis* (two strains), *Streptococcus salivarius*, *Streptococcus hemolyticus*, *Shigella dysentariae*, *Shigella paradysenteriae*, *Escherichia coli*, and *Aerobacter aerogenes*.

Those species that only slightly inhibited the growth of this flagellate were *Vibrio comma*, *Neisseria catarrhalis*, *Streptococcus pyogenes*, *Corynebacterium hofmannii*, and *Corynebacterium renale*. Bacteria that had no pronounced effect on the growth of *T. foetus* or which slightly accelerated the multiplication of this microorganism were *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Sarcina sp.*, and nine cultures of diphtheroids isolated from the genital tract of clinically normal
breeding bulls. *Brucella abortus* (strain 19) induced very rapid multiplication of the trichomonads but did not permit so long a survival time as the control cultures. One species, *Corynebacterium equi*, was outstanding in that it prolonged the life of *T. foetus* some 288 hours beyond the 240-hour limit of pure cultures of this trichomonad.

Bacterial filtrates and heat-killed cultures that were tested affected trichomonad growth in a manner comparable to the corresponding living bacteria cultured with *T. foetus*.

The probable nature of these accelerations and inhibitions has been discussed. It is believed that there are three main factors to be considered: (1) a change in the hydrogen ion concentration of the culture medium, (2) toxin production, and (3) release of additional nutrients favorable to trichomonad development.

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