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IN VITRO CULTIVATION OF THE TRICHOMONADIDAE: A STATE OF THE ART REVIEW

LOUIS S. DIAMOND

Abstract: The trichomonads of importance in human and veterinary medicine can all be cultivated in vitro. Most of them are easily axenized with the aid of antibiotics and migration techniques. Complex liquid media used for the axenic cultivation of trichomonads contain: (1) peptones - mixtures of peptides and amino acids prepared by hydrolytic treatment of proteins, (2) liver or yeast extracts serving as source of B vitamins, purines and pyrimidines, (3) maltose or glucose as the primary source of fermentable carbohydrates, (4) buffer, (5) reducing compounds, (6) small quantity of agar (0.01 – 0.05 %) to aid in maintaining a low redox potential, and (7) serum providing essential lipids and fatty acids. (8) Trace metals are most likely supplied as impurities of other medium components. Addition of iron supplement, however, favors the growth. A pH range 6.6 – 7.2 is suitable for the cultivation of most trichomonads, a pH 6.0 - 6.3 is optimum for *Trichomonas vaginalis*. Chemically defined media have been recently developed for the axenic cultivation of *T. vaginalis* and semi-defined media are available for *T. gallinae*, *Tritrichomonas foetus* and trichomonads from poikilothermic animals. Trichomonads are easy to plate in agar media if the incubation is carried out in anaerobic gas mixtures. Plating methods provide a useful tool for study of trichomonad nutrition, genetics, antigenic variation and drug susceptibility and their wider use is recommended. Further investigations are needed to deal with problems of cultivation. Advanced experimental methods would be of little use in absence of efficient means of growing the parasites in vitro. In addition, elucidation of the nutritional requirements of trichomonads, which can come about through the use and further development of chemically defined media, is prerequisite for targeted chemotherapy.

Key words: Trichomonadidae, cultivation, in vitro, xenic, axenic, defined media, cloning, plating.

INTRODUCTION

The trichomonads of greatest interest in human and veterinary medicine can all be cultivated xenically with a mixed bacterial flora. More importantly, with the exception of *Trichomonas tenax*, all of them are easily axenized, especially the three pathogenic species: *Trichomonas gallinae*, *T. vaginalis* and *Tritrichomonas foetus*.

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Since axenic cultures are the more versatile of the two types of cultures, these will be featured in this presentation.

Four issues will be addressed: 1) techniques of axenization, 2) the media used for axenic culture, 3) agar plating and cloning, and 4) the challenges of unsolved problems of axenic cultivation.

TECHNIQUES OF AXENIZATION

The natural habitats of trichomonads are also the habitats of a variety of bacteria and fungi. Therefore, the first problem to be dealt with in axenizing a trichomonad is to free it of its associated microorganisms.

The earliest workers taking advantage of trichomonad motility devised a variety of migration techniques to free their isolates of other microorganisms. The most widely used has been Glaser and Coria's 'V' tube technique first reported in 1935. I used a modification of the original design with great effectiveness (Diamond, 1957). Rees (1937) introduced the use of micromanipulation techniques to axenize *T. foetus.* The literature on these techniques has been reviewed (Diamond, 1983).

Modern workers have gravitated to the use of antibiotics and I highly recommend them with the proviso that their use be discontinued as soon as axenic growth has been attained. Antibiotics are not without effect on the trichomonads. Kott and Adler (1961) reported the antigenic types of two strains of *T. vaginalis* changed after treatment of the cultures with antibiotics. Chloramphenicol can be toxic for *T. vaginalis* (Honigberg, 1978). Stabler et al. (1964) reported the use of streptomycin resulted in attenuation of virulence of *T. gallinae.*

Each investigator has a special preference regarding the choice of antibiotics. I use a mixture of penicillin, 1 000 units/ml, and streptomycin sulfate, 1 μg/ml, supplemented with kanamycin or gentimycin, 50 μg/ml to erradicate both bacteria and mycoplasma, and mycostatin 100 μg/ml to rid the culture of fungi. Fungi can also be eliminated by culturing the trichomonads xenically for several subcultures before attempting axenization. The fungi cannot compete with the bacteria present in the culture and are eliminated.

Xenic cultures serve frequently as a source of trichomonads for the initiation of axenic cultures. The only exception to this, as far as I am aware, is *T. tenax.* I was able to establish axenic cultures of this species only with monoxenically cultivated organisms (Diamond, 1962).

Axenic cultures of the three pathogens, *T. gallinae, T. vaginalis* and *T. foetus,* can be and are most frequently initiated by introducing infected host material directly into media used for axenic culture. In fact, the ease with which axenic cultures of these species can be initiated has led to increasing use of axenic culture techniques for the diagnosis of infection.

Xenic Cultures

Many media are available for this purpose
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(1934) Ringer's Solution-Dried Blood Serum
(1925) LES Medium. The latter, known to
to be used successfully for the xenic cultivatio
by Boeck (1924) for cultivation of intestina

Axenic Cultures

The media used for this purpose can be
semi-defined, and chemically defined.

Undefined media

The undefined media are the most widely
position. The major ingredients are shown

Table 1. *Major ingredients of undefined media for*

1. Peptone
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6. Agar
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2) A liver or yeast extract is always p
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3) Maltose or glucose serve as the prim

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MEDIA

Xenic Cultures

Many media are available for this purpose, some specifically developed for cultivation of flagellates (consult TAYLOR and BAKER, 1968). I recommend two - RAKOFF'S (1934) Ringer's Solution-Dried Blood Serum medium, and BOECK and DRBOHLAV'S (1925) LES Medium. The latter, known to most investigators as the first medium to be used successfully for the xenic cultivation of *Entamoeba histolytica*, was devised by BOECK (1924) for cultivation of intestinal flagellates.

Axenic Cultures

The media used for this purpose can be divided into three types - Undefined, semi-defined, and chemically defined.

Undefined media

The undefined media are the most widely used, and are strikingly similar in composition. The major ingredients are shown in Table 1. They are discussed below.

Table 1. *Major ingredients of undefined media for axenic culture of Trichomonads*

1. Peptone
2. Liver/Yeast
3. Carbohydrate
4. Salts/Buffers
5. Reducing agents
6. Agar
7. Serum
8. Trace metals

1) A common feature of these media is the presence of one or more peptones, e.g., Trypticase, an enzymatic digest of casein, or Bacto Peptone, an enzymatic digest of beef muscle. These consist principally of mixtures of peptides and amino acids, the proportions of the constitutents being dependent on the intensity of the hydrolytic treatment to which the original substrates have been subjected. The harsher and longer the treatment, the greater the amino acid content and the less the peptide content, and viceversa. These peptones are also a source of trace metals. Trypticase is a specially rich source of amino sugars.

2) A liver or yeast extract is always present. In some media in which liver is incorporated, peptones may not be added. Liver itself is rich in amino acids. Both the liver and yeast serve primarily as sources of B vitamins, purines and pyrimidines.

3) Maltose or glucose serve as the primary source of fermentable carbohydrate.

4) Buffering of the medium is usually accomplished through use of sodium and/or potassium phosphate salts. I prefer combinations of the latter. Sodium chloride may be added as a constituent of the medium. In other cases it is provided as a contaminant of the biological products in the medium.

5) All of the media contain reducing agents. Cysteine is the most commonly used. Thioglycollate and thiomalic acid have been used, but to a lesser extent. I have had unusual success with a combination of cysteine and ascorbic acid.

6) Agar is often added to the media used in axenic cultivation of trichomonads. When it is, it is used in very small quantities, from 0.01 to 0.05 %. Agar is added primarily to aid in maintaining a low redox potential. In addition, I suspect that it provides binding sites for the nutrients in the medium and in this way aids the growth of the flagellates. In attempting isolation of a trichomonad directly in axenic medium, I make it a practice to inoculate medium with and without the addition of agar.

7) Serum is an essential ingredient of these media. It provides a host of nutrients, among them lipids, fatty acids, amino acids and trace metals. It also acts as a detoxifying agent. Cattle, horse and sheep have been the most widely used sources of serum. SAMUELS and BEIL (1962) replaced serum with cream for axenic cultivation of several trichomonads in undefined media.

8) Except for iron, nothing is known about the trace metal requirements of trichomonads (consult PETERSEN and ALDERETE, 1984, and GORELL, 1985). HOLLANDER (1976) was probably the first to add an iron supplement to a medium for the cultivation of trichomonads. The iron was in the form of a sulfate and was added to a modification of DIAMOND's (1957) TYM medium. Dr. C. F. T. MATTERN (personal communication) reported having unusual success isolating *T. vaginalis* directly from clinical material using TYI-S-33 medium devised by DIAMOND et al. (1978) for axenic culture of *Entamoeba histolytica*. One reason for Mattern's success may be that this medium is supplemented with iron in the form of ferric ammonium citrate. Other trace metals, for which the requirements are unknown, are most likely supplied as impurities of other components of the medium.

A pH ranging from 6.6 to 7.2 is acceptable for the cultivation of most trichomonads. A pH of 6.0 to 6.3 favors the growth of *Trichomonas vaginalis*.

The two most widely used undefined media for cultivation of trichomonads are: CPLM (JOHNSON and TRUSSELL, 1943) devised specifically for the cultivation of *T. vaginalis*, but used for the culture of several trichomonads; and TYM (DIAMOND, 1957), developed for the growth of a wide variety of Trichomonadidae. The most recent addition to the list of these flagellates grown in TYM is *Tritrichomonas muris*. SAEKI et al. (1983) succeeded in growing this flagellate axenically in TYM supplemented with an autoclaved murine cecal extract.

Refinement of the available undefined media can be expected to result in improved yields of flagellates, but for most research purposes acceptable levels of growth have been attained. SCHWARTZ (1980) substituted Loeffler's dried blood serum for

the fresh whole serum used in TYM a
this modification. Flagellate yields were
supplemented medium.

Semi-defined media

Several of these media have been de
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Chemically-Defined Media

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AGAR PLATING TE

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the fresh whole serum used in TYM and succeeded in culturing *T. vaginalis* with this modification. Flagellate yields were equal to those obtained with the fresh serum supplemented medium.

Semi-defined media

Several of these media have been developed. Among them can be listed the SL medium of SHORB and LUND (1959), and SLM of LUND and SHORB (1962) for the cultivation of *T. gallinae*, *T. vaginalis* and *T. foetus*; media 5E and 7E devised by LEE et al. (1962) for cultivation of trichomonads of poikilothermic hosts; and LINSTEAD'S (1981) modified CMRL 1066 for *T. vaginalis*. Recently, WANG et al. (1984) described a semi-defined medium, HUT, for *T. foetus*.

Chemically-Defined Media

The credit for developing the first chemically defined media for *Trichomonas* goes to LINSTEAD (1981), who described DL 7 and DL 8 for the axenic cultivation of *T. vaginalis*. Especially noteworthy was the replacement of serum, always a stumbling block in developing a defined medium, with a mixture of bovine albumen Fraction V, cholesterol, and GPOS (DL-Glyceryl-L-Palmitate-2-oleate-3 stearate).

AGAR PLATING TECHNIQUES AND CLONING

The ability to grow cells, be they protozoa, bacteria, or tumor cells, as discrete colonies on agar plates provides a powerful tool for the study of such diverse cellular attributes as their nutritional requirements, genetics, antigenic variation, and drug sensitivity. With care the techniques can be used for cloning of wild populations. In the last regard, whereas generations of microbiologists have depended on agar plating for cloning, many protozoologists remain skeptical believing that the only way to clone a population is to do it visually using the microscope. Of course the protozoologist has an advantage over the microbiologist, he can visualize a single organism much easier than can the latter. KULDA and SERBUS (1968) described a method for cloning trichomonads in which the flagellates were spread on a thin layer of agar and isolated by a hollow puncture knife attached to a microscope objective. This device permitted direct observation of the cell to be isolated.

Several techniques have been devised for plating trichomonads. Some involve the use of a single agar layer, others a double layer. In the case of the latter, the bottom layer is a hard nonnutritive supporting agar, and the top layer a soft nutritive agar inoculated with the assay organisms. Incubation is carried out under anaerobic conditions. Atmospheres of carbon dioxide, hydrogen and even illuminating gases have been used. Consult DIAMOND (1983) for a review of the literature and detailed descriptions of recommended techniques.

Despite the fact that the trichomonads are relatively easy to plate, only a handful of investigators have made use of this technology. Agar plating techniques have been employed to study drug sensitivity (FILADORO and ORSI, 1958, SAMUELS and STOUDER, 1962, and WEST et al., 1962), for cloning (IVEY, 1961, SAMUELS, 1962), for study of colony morphology (HOLLANDER, 1976), for the assessment of hemolytic activity as an indicator of virulence (KRIEGER et al., 1983), and most recently for isolation of mutants of *T. foetus* in the study of purine and pyrimidine metabolism (WANG et al., 1984).

UNSOLVED PROBLEMS IN AXENIC CULTIVATION: THE CHALLENGES

Relatively few of the described Trichomonadidae have been cultured axenically. Moreover, not all of those which have been cultivated xenically, have been cultured axenically. The availability of such cultures would promote the study of the biochemistry, physiology, immunology, etc., of these parasites. Information so gained would add materially to our knowledge of primitive eukaryotes.

Challenge 1

Develop methods which will enable axenic cultivation of more species. I am of the opinion that the basic media to accomplish this are available. They may need modification. For example, *T. muris*, a species refractory to all attempts to grow it in vitro, was finally cultivated, and axenically at that, in TYM supplemented with an autoclaved murine cecal extract (SAKEI et al., op. cit.). As noted earlier, I was able to axenize *T. tenax* from monoxenic cultures, but not from clinical material or xenic culture (DIAMOND, 1962).

Knowledge of the nutritional requirements of an organism and the pathways involved in the metabolism of the nutrients are prerequisite for targeted chemotherapy at the molecular level. Nowhere is this better illustrated than by the work of BACCHI and his colleagues who have been able to control the growth of certain african trypanosomes not only in vitro, but also in vivo by intervention of polyamine metabolism with the inhibitor of ornithinedecarboxylase, DFMO (D, L-Difluormethyl-ornithine) (MCCANN et al., 1983).

Challenge 2

Develop chemically defined media in which serial subculture can be achieved. Elucidation of the nutritional requirements of trichomonads can come about only through use of such media. As noted earlier, LINSTEAD has developed two such media for *T. vaginalis*. WANG et al. (op. cit.) are close to developing a chemically defined medium for *T. foetus*. One is needed for *T. gallinae*. This parasite still remains a cause of serious economic loss among poultry raisers.

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ULTIVATION: THE CHALLENGES

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Above all else, we need more young investigators to take up these challenges and deal with the problems of cultivation. I am fully aware that today the gene is the thing, and if you are not seen carrying a gel or an autoradiographic plate in your hand you are not one of the in group. But, you cannot study and exploit the genes of a trichomonad if you do not have the means of growing it in vitro.

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IMMUNOLOGIC AND BIOCHE IDENTIFICATION OF TR SPECIES AN

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Abstract: Classical animal taxonomy has alw
should probably continue to be so. Biochemical
firstly because microscopic identification of p
objective tests, and secondly to help resolve pr
The necessary biochemical and immunological
find no difficulty in converting them into useful

Key words: *T. vaginalis*, taxonomy, pathogen

INTROD

Classical animal taxonomy is based
years a satisfactory definition of a species
1962): »A community whose distinctive m
of a competent systematist, sufficiently d
definition is in principle clearly unsound
but in practice it often works very well.
which appear to be morphologically alm
has led to a switch of emphasis towards
interbreed; and a current definition mig
potentially interbreeding populations wh
such groups« (SAVORY, 1962). This newe
in many areas, including any of the pro
life cycle; and for these and many other c
criteria will remain paramount. In our o

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