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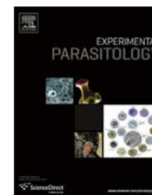

Axenization and optimization of *in vitro* growth of clonal cultures of *Tetratrichomonas gallinarum* and *Trichomonas gallinae*

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A rapid and simple procedure was established to obtain clonal axenic cultures of *Tetratrichomonas gallinarum* and *Trichomonas gallinae* and to optimize their *in vitro* growth conditions. Medium 199 was used for axenization of two genetically different clones of *T. gallinarum* and *T. gallinae*. Six different media were used to optimize the growth behaviour of axenically grown parasites: Medium 199, TYM, TYI-S-33, Hollander fluid (HF), *Trichomonas vaginalis* (TV) and modified TV media. The highest cell yields for both axenic clones of *T. gallinarum* were obtained in modified TV medium without antibiotics. The maximum numbers of trophozoites of *T. gallinae* were obtained in an optimized HF medium. This study demonstrated that axenic cultures for *T. gallinarum* and *T. gallinae* could be obtained avoiding the migration technique through a V-tube. Following axenization and optimization, both clones of *T. gallinarum* and *T. gallinae* could be propagated both aerobically and anaerobically.

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1. Introduction

Tetratrichomonas gallinarum is a parabasalid flagellate with a world-wide distribution commonly inhabiting the intestinal tract of poultry (Honigberg, 1978). Furthermore, this protozoon can also be detected in the liver of chickens and turkeys (Grabensteiner and Hess, 2006). Even though the parasite was already described almost one hundred years ago by Martin and Robertson (1911), the contribution to pathological changes in its host is remains controversial (Tyzzer, 1934; Allen, 1941). Some of this debate can be attributed to the frequent occurrence of the parasite in mixed infections with different protozoa, especially with *Histomonas meleagridis* (Allen, 1936). *Trichomonas gallinae* is a flagellated protozoon affecting the upper digestive tract and various internal organs of different avian species especially columbiformes (doves and pigeons) and falcons (Stabler, 1954). The *in vitro* propagation of trichomonads can be done by inoculation of infected material from the oral cavity (*T. gallinae*) (Honigberg, 1978) or the intestine (*T. gallinarum*) (Allen, 1941) of infected birds into a suitable growth medium. So far, different media and techniques have been described to obtain axenization of such cultures, but most of them are rather laborious and time consuming (Diamond, 1957; Kulda et al., 1974). The *in vitro* growth of *H. meleagridis* and *T. gallinarum* in Medium 199 containing high numbers of protistis under xenic conditions was described by Hess et al. (2006). However, in their experiment there was no attempt to produce high numbers of *T. gallinarum* under axenic conditions in Medium 199. The development of axenic mono-eukaryotic cultures of *T. gallinarum* and *T. gallinae* would be of importance to create a source of pure flagellates for biological and biochemical studies under well-defined conditions. Thus, the objectives of this study were to establish a fast and simple procedure to obtain axenic cultures of *T. gallinarum* and *T. gallinae* and to optimize the *in vitro* growth conditions of these parasites in order to get high cell yields.

2. Materials and methods

2.1. Clonal cultures

2.1.1. Tetratrichomonas gallinarum

The clonal cultures of *T. gallinarum*, labelled *T. gallinarum*/Turkey/Austria/2721-C7/03 (briefly, clone 2721-C7/03) and *T. gallinarum*/Turkey/Germany/4114-C5/05 (clone 4114-C5/05) were used in this study for axenization and optimization of the *in vitro* growth. Both isolates were established from the caecal content of a turkey from flocks located either in Austria (clone 2721-C7/03) or in Germany (clone 4114-C5/05) suffering from severe histomonosis. The *in vitro* isolation, propagation and establishment of clonal cultures were done as described recently by Hess et al. (2006). The clones were analysed genetically by sequencing the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 after performing PCR with the primers TFR1 (5′-TGC TTC AGT TCA GCG GGT CTT CC-3′) and TFR2 (5′-CGG TAG GTG AAC CTG CCG TTG G-3′) according to the protocol described

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by Felleisen (1997). Using GenBank™, the particular isolates showed complete identity with two different *T. gallinarum* strains published in the work of Cepicka et al. (2005). The sequence of clone 2721-C7/03 resulted in a product size of 329 bp and was identical with that of strain KROC (GenBank Accession No. AY245147), whereas clone 4114-C5/05 (insert 335 bp) could be allocated to strain GPO (GenBank Accession No. AY245129).

2.1.2. Trichomonas gallinae

Two different cultures of *T. gallinae* named *T. gallinae*/Budgerigar/Austria/5895-C1/06 (clone 5895-C1/06) and *T. gallinae*/Pigeon/Austria/7895-C2/06 (clone 7895-C2/06) were used for axenization and optimization of the *in vitro* growth in this study. Both isolates were established from oral swabs of a budgerigar and a pigeon, respectively. The same phylogenetic analyses were performed as above for *T. gallinarum*. The nucleotide sequence (300 bp) of clone 5895-C1/06 was identical to strain g7 (GenBank Accession No. AY349182) published in the work of Kleina et al. (2004). Sequence of clone 7895-C2/06 was so far not available in GenBank. The fragment of 296 bp showed complete identity with a recently submitted sequence of *Trichomonas gallinae* (clone 7895-C1/06) with the Accession No. FN433474.

2.2. Culture media

Six different media were used in this study: (1) Medium 199 + Earlés salts + L-Glutamine + 25 mM HEPES + L-Amino acids (Gibco™) supplemented with 15% fetal calf serum (FCS), (2) Trypticase Yeast extract Maltose medium (TYM medium), (3) Trypticase Yeast extract Iron-Serum-33 medium (TYI-S-33 medium), (4) Hollander fluid medium (HF medium), (5) *Trichomonas vaginalis* medium (TV medium) and (6) modified *Trichomonas vaginalis* medium (modified TV medium). The modified TV medium contained only half amount of dibasic potassium phosphate (K_2HPO_4) and monobasic potassium phosphate (KH_2PO_4). TYM medium was prepared according to the protocol of Diamond (1983), TYI-S-33 medium according the protocol of Diamond et al. (1978). HF medium with 1 g of microbiology agar–agar per 950 ml of distilled water (Merck, Darmstadt, Germany) was prepared according to the protocol of Smith (1983), but either FCS or chicken serum was used instead of pooled human serum. FCS was used because it was already included in Medium 199 before axenization and chicken serum was used for comparison. The protocol of Gentry et al. (1985) was followed for the preparation of TV medium, but bovine serum was replaced either by FCS or chicken serum.

TV medium is composed as follows: 80 mg of K_2HPO_4, 80 mg of KH_2PO_4, 2 g of casine digest, 1 g of yeast extract, 1 g of dextrose, 200 mg of NaCl, 200 mg of L-cysteine hydrochloride monohydrate, 20 mg of ascorbic acid, 2.28 mg of ferric ammonium citrate and 10% of FCS or chicken serum per 100 ml of distilled water. The pH level for all investigated media was adjusted to 7.2 before sterilization at 121 °C for 15 min. After autoclaving, either FCS or chicken serum was added and the medium was dispensed in 9 ml aliquots in 50 ml tubes (Sarstedt, Nümbrecht, Germany). All media were kept at −20 °C and brought to room temperature before use. Additionally, an antifungal (2.5 μg amphotericin B/ml medium) was added to Medium 199 before use.

2.3. Bacteriological investigation

Bacteriological investigations were made from xenic *T. gallinarum* and *T. gallinae* cultures initially grown in Medium 199. For this, 100 μl of cultures material were streaked out onto Columbia agar supplemented with 5% sheep blood (COS) (BioMérieux, Vienna, Austria), MacConkey agar (McC) (BioMérieux, Vienna, Austria), Schaedler agar with 5% sheep blood (SCS) (BioMérieux, Vienna, Austria) and Sabouraud Gentamycin Chloramphenicol agar (SGC) (BioMérieux, Vienna, Austria). Except the SGC agar and the SCS, all agar plates were incubated at 37 °C aerobically for 24 h. The SGC agar plates were incubated at 42 °C aerobically for 48 h, the SCS plates were incubated at 37 °C anaerobically for 24 h. Based on the results of the bacteriological findings sensitivity tests according to Bauer et al. (1966) were done. The following antibiotic discs were used: meropenem 10 μg, penicillin G 10 U, neomycin 30 μg, streptomycin 10 μg, doxycyclin 30 μg, tilmycosin 15 μg, colistinsulphate 10 μg, amoxycillin 2 μg, enrofloxacin 5 μg, marbofloxacin 5 μg, tylosin 30 μg and spectinomycin 25 μg. The bacteriological investigations of all cultures containing antibiotics were done daily starting 24 h after the first treatment. The cultures were passaged every 48 h into fresh media adding antibiotics.

After axenization the cultures of all protozoa were transferred and propagated in other types of media namely: TYM medium, TYI-S-33 medium, HF medium, TV medium and modified TV medium. Bacteriological investigations as described above were performed to confirm axenization. The growth studies began after two passages of these axenic clones in all types of media tested.

2.4. Growth studies of axenic T. gallinarum and T. gallinae cultures

Prior to optimization of growth behaviour under aerobic or anaerobic conditions within the different media, a comparison between the cell yields at 40 °C and 37 °C was performed to investigate the influence of temperature on the different clones. To study the effect of aerobic and anaerobic conditions on the growth of the axenic *T. gallinarum* and *T. gallinae* cultures an anaerobic environment was produced using GENbox anaer bags in anaerobic jars (BioMérieux, Vienna, Austria). The initial concentration was 10^5 cells for each culture. Additionally, the influence of the antibiotics streptomycin and penicillin on the growth behaviour of both protozoa was tested. For this purpose a mixture of 120 μg streptomycin and 120 international units (IU) penicillin per ml medium was added to the media. All types of treatment in the different culture media were incubated at 37 °C and 40 °C.

To examine the effect of the different growth conditions, live trophozoites from the cultures were counted using a haemocytometer. Samples were mixed with an equal amount of trypan blue 0.4% (Invitrogen, Vienna, Austria) to differentiate between live and dead trophozoites. The counting was performed every 24 h until live flagellates disappeared from the culture. Each growth study was performed in triplicate and triplicate counts of trophozoites were done for each sample as well. The mean of these counts was used for evaluation of the growth behaviour of the protozoa. The *t*-test for unpaired samples was used for statistical analysis.

3. Results

3.1. Bacteriological investigation

From the xenic cultures the following bacteria were isolated: *Escherchia coli*, *Proteus* sp. and *Streptococcus* sp. *E. coli* was sensitive to meropenem, neomycin, streptomycin and colistin. *Proteus* sp. was sensitive to meropenem and neomycin. *Streptococcus* sp. was sensitive to meropenem, neomycin, streptomycin, and penicillin.

As all bacteria showed sensitivity to meropenem it was the first choice for inactivation of prokaryotic organisms in all protozoan cultures. A negative effect on growth of trophozoites of both *T. gallinarum* and *T. gallinae* was found when meropenem was used in concentrations ranging from 100 μg/ml to 12 μg/ml. Using 6 μg/ml meropenem did not influence the growth of the flagellates, but this concentration was too low to kill *Streptococcus* sp. completely. It was found that an antibiotic mixture of meropenem

(6 μg/ml), streptomycin (200 μg/ml) and penicillin (200 IU/ml) was able to kill all species of bacteria. Following this procedure no bacteria could be recovered after three passages and bacteriological examinations remained negative even in the absence of antibiotics.

3.2. Growth studies of axenic *T. gallinarum* cultures

All optimization was done at 40 °C as higher cell yields were obtained than at 37 °C (data not shown). The growth curves of both clones of *T. gallinarum* cultivated without antibiotics in Medium 199, TYM medium, TYI-S-33 medium, HF medium (with or without agar) and TV medium are presented in Fig. 1(A–D).

3.2.1. Medium 199

Under aerobic conditions a pronounced decrease in the number of trophozoites of both *T. gallinarum* clones was already present 24 h after beginning incubation and no viable trophozoites were detected at 48 h. In contrast, under anaerobic conditions viable trophozoites were found up to 144 h after initial inoculation in the presence of antibiotics and until 168 h in absence of antibiotics (clone 2721-C7/03). For clone 4114-C5/05 live flagellates were detected for 72 h either in the presence or absence of antibiotics. In general, the growth rate of trophozoites in Medium 199 was much lower compared with the other media investigated.

3.2.2. TYM medium

Viable trophozoites from clone 2721-C7/03 were detected for 144 h under all growth conditions. For clone 4114-C5/05 flagellates multiplied for 96 h under aerobic and for 120 h under anaerobic conditions. Higher numbers of trophozoites were obtained when omitting antibiotics but this effect was not significant ($P > 0.05$).

3.2.3. TYI-S-33 medium

No viable trophozoites were detected after 216 h (clone 2721-C7/03) and after 192 h (clone 4114-C5/05) under aerobic conditions. The cells of both clones could be amplified for 144 h under anaerobic conditions and higher numbers of trophozoites were gained in the absence of antibiotics ($P > 0.05$).

3.2.4. HF medium with or without agar

The trophozoites of clone 2721-C7/03 were able to grow under aerobic and anaerobic conditions with or without antibiotics for 144 h. Clone 4114-C5/05 was detected in the absence of antibiotics under aerobic conditions for 144 h and for 120 h under anaerobic conditions. Under both aerobic and anaerobic conditions viable trophozoites of clone 4114-C5/05 could be found for 120 h in the presence of antibiotics. The addition of agar is not essential for the growth of *T. gallinarum*, as both clones of this species were able to grow in HF medium without agar in a higher number than in HF medium supplied with agar.

3.2.5. TV medium

Under aerobic and anaerobic conditions with and without antibiotics viable trophozoites of clones 2721-C7/03 and 4114-C5/05 could be found up to 96 h and 120 h, respectively. The highest growth rate of cells from clone 2721-C7/03 was 1.02×10^7/ml under aerobic conditions and 8.97×10^6/ml under anaerobic conditions in the absence of antibiotics. In contrast, the maximum number of trichomonads of clone 4114-C5/05 was 7.1×10^6/ml and 7.8×10^6/ml under aerobic and anaerobic conditions, respectively, when TV medium was prepared without antibiotics.

3.2.6. Modified TV medium

The highest trophozoite counts under all incubation conditions were observed with modified TV medium. Modified TV medium also supplied viable protozoa for a longer period than TV medium and the proliferation rate was not affected by the incubation conditions. The highest number of trophozoites from clone 2721-C7/03 was 1.3×10^7/ml under aerobic conditions and 1.2×10^7/ml under anaerobic conditions without the addition of antibiotics. While, the maximum growth level for clone 4114-C5/05 was 9.4×10^6/ml under both conditions.

The rate of growth of *T. gallinarum* in TV medium and modified TV medium containing FCS is shown in Fig. 2(A–D). There was no significant difference ($P > 0.05$) in the growth rate of each clone

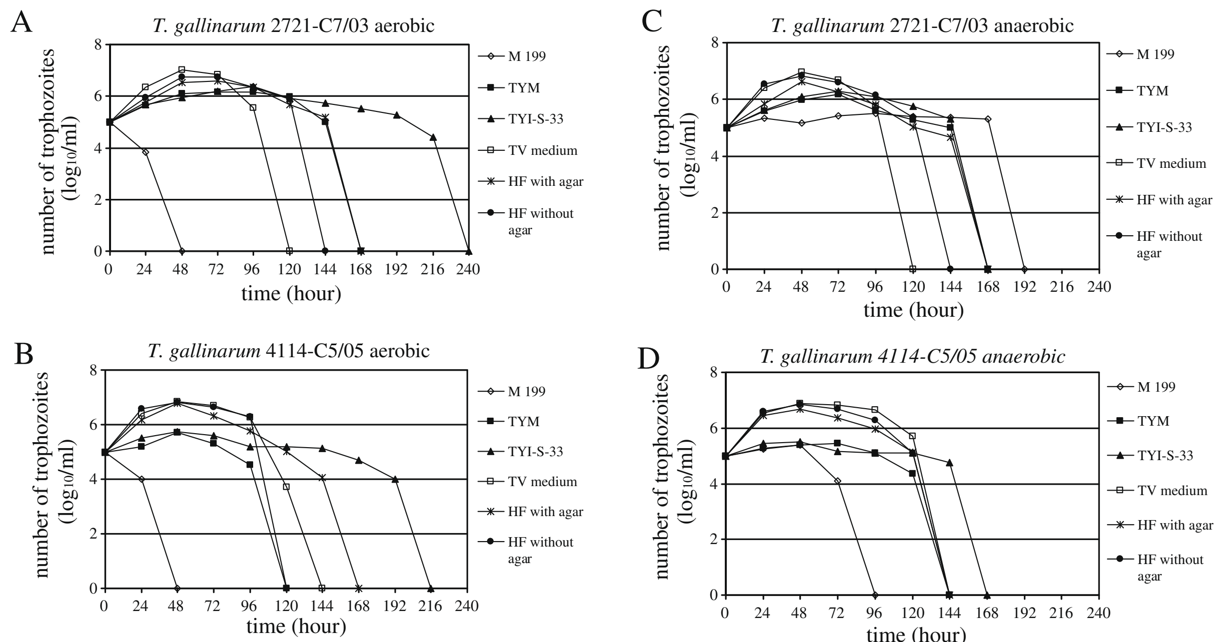

Fig. 1. Growth curves of *T. gallinarum* clones 2721-C7/03 and 4114-C5/05 in different media without antibiotics, under aerobic (A + B) and anaerobic (C + D) conditions.

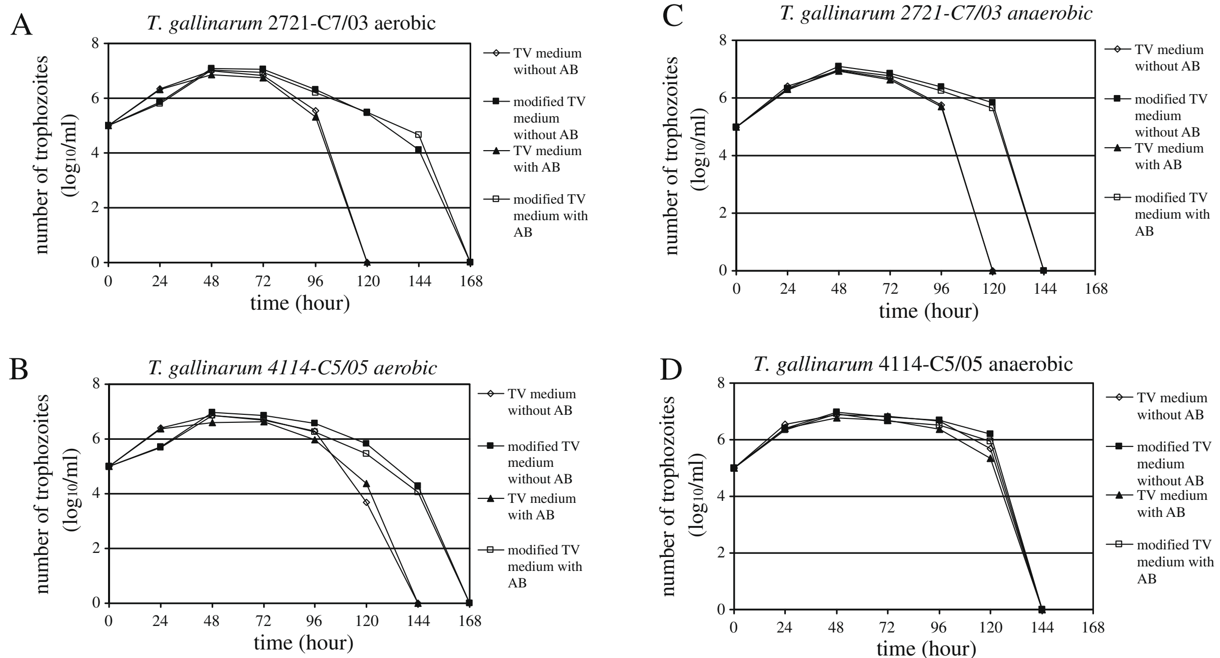

Fig. 2. Propagation of *T. gallinarum* in TV medium and modified TV medium with FCS in the presence or absence of antibiotics (AB) for both clones of 2721-C7/03 and 4114-C5/05, under aerobic (A + B) and anaerobic (C + D) conditions.

of *T. gallinarum* in both TV medium and modified TV medium under all conditions.

3.2.7. The effect of chicken serum on the growth of T. gallinarum

The presence of chicken serum in HF medium (with or without agar), TV medium and modified TV medium resulted in lower cell yields than with the same media containing FCS (data not shown).

3.3. Growth studies of axenic cultures of T. gallinae

Optimal growing temperature of *T. gallinae* was found to be 37 °C instead of 40 °C (data not shown). As a result, the optimization of growth conditions was performed at 37 °C. The aerobic and anaerobic growth curves of both clones in Medium 199, TYM medium, TYI-S-33 medium, TV medium, modified TV medium and HF medium with agar but without antibiotics for all media are presented in Fig. 3(A–D).

3.3.1. Medium 199

Viable trophozoites of clone 5895-C1/06 were found for 168 h under aerobic conditions and for 144 h under anaerobic conditions in the presence or absence of antibiotics. Furthermore, under aerobic conditions cells from clone 7895-C2/06 could be detected for 216 h in cultures independent from antibiotics. In contrast, live trophozoites would be found for only 168 h where anaerobic incubation was used. No influence on the growth of *T. gallinae* could be detected with regard to the use of antibiotics, as no significant difference was detected ($P > 0.05$).

3.3.2. TYM medium

No cells from both clones (5895-C1/06 and 7895-C2/06) of *T. gallinae* were observed in any of the tubes containing TYM medium during the whole investigation period (264 h) under all growth conditions.

3.3.3. TYI-S-33 medium

No viable trophozoites were detected after 120 h (clone 5895-C1/06) and after 240 h (clone 7895-C2/06) under aerobic condition with or without antibiotics. The trophozoites of clone 5895-C1/06 were grown for 72 h and clone 7895-C2/06 for 144 h under anaerobic condition in the presence or absence of antibiotics. However, in the absence of antibiotics a higher number of trophozoites were obtained ($P > 0.05$).

3.3.4. TV medium

Under aerobic and anaerobic conditions viable trophozoites of clone 5895-C1/06 could be found until 120 h independent of the use of antibiotics. Live cells of clone 7895-C2/06 were noticed for 168 h in the absence of antibiotics and for 144 h in the presence of antibiotics. Under anaerobic conditions the trophozoites were detected for 192 h in the presence or absence of antibiotics.

3.3.5. Modified TV medium

The trichomonads from clone 5895-C1/06 could be found until 144 h under both aerobic and anaerobic conditions with or without antibiotics. Clone 7895-C2/06 trophozoites were detected for 192 h after aerobic incubation and for 120 h under anaerobic conditions regardless of the presence or absence of antibiotics.

3.3.6. HF medium with or without agar

Aerobic and anaerobic environment yield in viable flagellates of clone 5895-C1/06 and clone 7895-C2/06 could be found until 192 h and 144 h, respectively, independent from the use of antibiotics in HF medium with agar. In HF medium without agar *T. gallinae* was able to grow to higher numbers than in HF medium containing agar under all incubation conditions as shown in Fig. 4(A–D). The maximum number of cells counted for clone 5895-C1/06 was 5.9×10^6/ml under aerobic incubation in the absence of antibiotics. The highest cell yields for clone 7895-C2/06 were 1.1×10^7/ml following anaerobic incubation without antibiotics. Although the growth rate of the two different clones in HF medium with

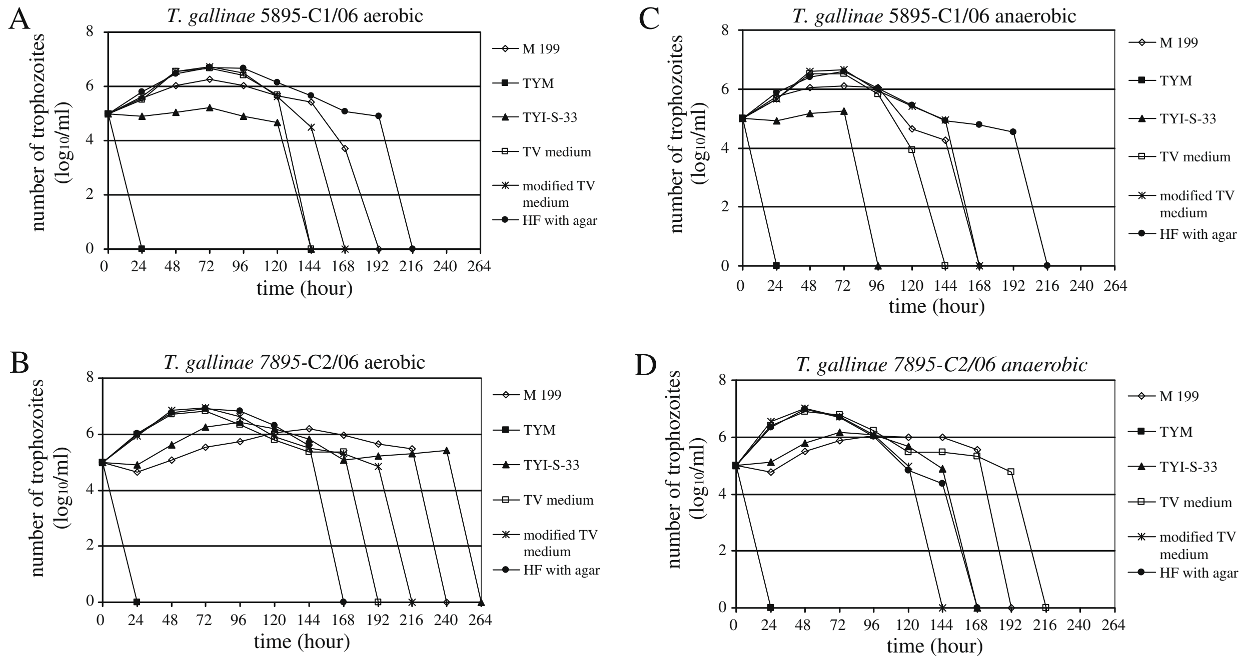

Fig. 3. Growth curves of *T. gallinae* clones 5895-C1/06 and 7895-C2/06 in different media without antibiotics, under aerobic (A + B) and anaerobic (C + D) conditions.

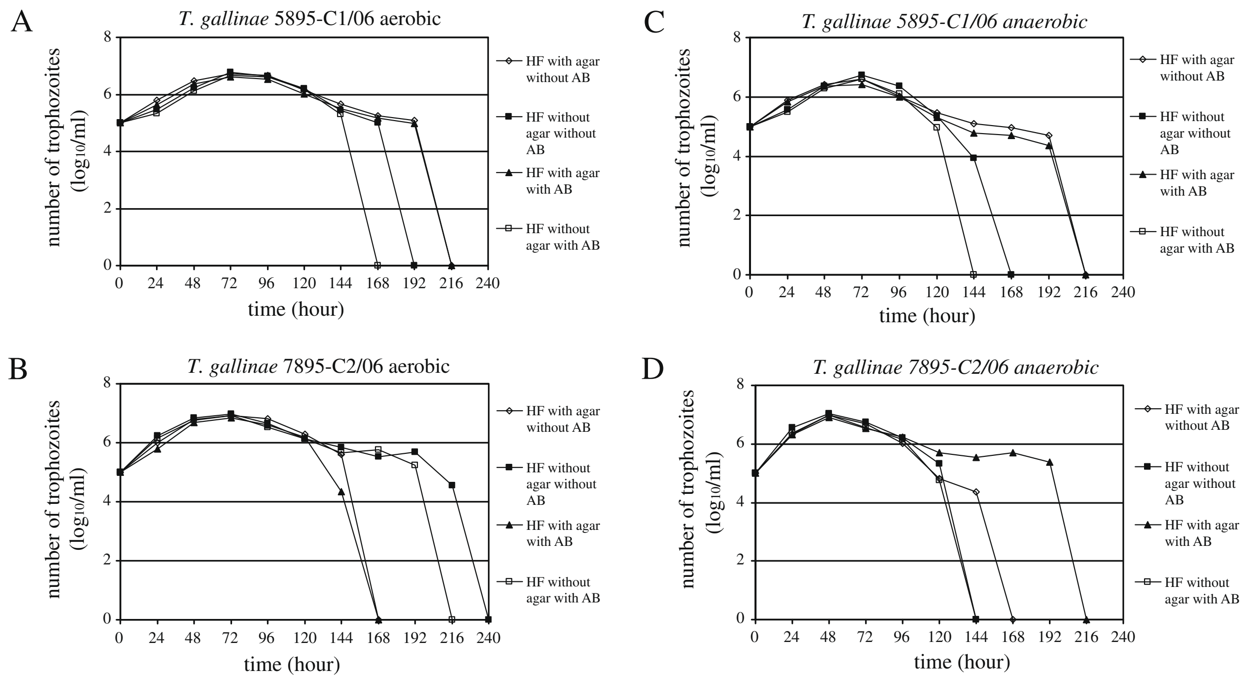

Fig. 4. Comparison between HF medium with and without agar containing FCS in the presence or absence of antibiotics (AB) for both clones of *T. gallinae* 5895-C1/06 and 7895-C2/06, under aerobic (A + B) and anaerobic (C + D) conditions.

or without agar varied, no significant difference ($P > 0.05$) in the cell number could be determined under all conditions.

3.3.7. The effect of chicken serum on the growth of T. gallinae

The cell yields in TV medium, modified TV medium and HF medium (with or without agar) containing chicken serum were lower than those for the same media containing FCS (data not shown).

4. Discussion

Available data on axenic propagation and growth behaviour of *T. gallinarum* and *T. gallinae* is very limited. Axenization and propagation of both trichomonad species provides a source of pure flagellates for biological and biochemical studies of the parasites. In this study, procedures were established to obtain high yields of axenically grown clonal cultures of *T. gallinarum* and *T. gallinae*.

In earlier studies, axenic cultures of *T. gallinarum* and *T. gallinae* were obtained using the V-tube migration technique in TYM medium (Diamond, 1957; Kulda et al., 1974). However, this technique is tedious and time consuming (Diamond, 1962). In this investigation 1.0×10^5 trophozoites were used to evaluate the growth behaviour of *T. gallinarum* and *T. gallinae* in axenic cultures under different incubation schemes following a suggestion of Diamond (1957), who noticed that between 5.0×10^4 and 2.0×10^5 cells of trichomonads were required to maintain axenic cultures.

Undoubtedly, there are various factors which may influence the growth behaviour of flagellates *in vitro* and only selected parameters could be addressed within such a study.

In this work the optimal temperature for *T. gallinarum* was 40 °C with higher cell yields than those observed at 37 °C confirming the finding of Theodorides (1964). In contrast, the number of live cells recorded for *T. gallinae* was higher at 37 °C than at 40 °C, as reported by others (De Carli et al., 1996; De Carli and Tasca, 2002; Tasca and De Carli, 2003). These findings obviously reflect the clinical environment of both parasites, either in the pharynx or the caeca of birds.

For appropriate *in vitro* propagation of human trichomonas, usually large numbers of essential nutrients including carbohydrates, amino acids, purines, pyrimidines, fatty acids, vitamins, and iron are mandatory as demonstrated by Linstead (1989). Furthermore, TV medium, modified TV medium and HF medium (with or without agar) contain essential ingredients such as casein, yeast extract, carbohydrate (dextrose in both TV medium and modified TV medium or maltose in HF medium), iron, monobasic, and dibasic potassium phosphate. In this context, it should be mentioned that iron was defined, as an essential nutritional source for trichomonads (Lehker et al., 1991). Furthermore, ascorbic acid is described as beneficial for the growth of *T. gallinae* (Stabler, 1954). The absence of iron and the lower amount of ascorbic acid in TYM medium could be an explanation for the very limited success to grow both clones of *T. gallinae* in this medium, as described earlier (Diamond, 1957).

An important result of this work was that the highest numbers of *T. gallinarum* were detected in modified TV medium followed by TV medium. The advantage of TV media could be explained by the presence of dextrose which is the most rapidly utilized sugar by *T. gallinarum* (Theodorides, 1964). The maximum number of *T. gallinarum* in modified TV medium for clone 2721-C7/03 was 1.3×10^7/ml and for clone 4114-C5/05 was 9.4×10^6/ml, while the highest number for *T. gallinarum* in axenic condition obtained by Theodorides (1964) was about 1.0×10^7/ml in Diamond's medium containing 10% fresh sheep serum.

In comparison, the best growth of *T. gallinae* was found in HF medium, which could be attributed to the presence of maltose as recorded by Daly (1970). He stated that the growth rate of *T. gallinae* was higher in media supplemented with maltose than other carbohydrates. The maximum number for *T. gallinae* clone 5895-C1/06 was 5.9×10^6/ml and for clone 7895-C2/06 was 1.1×10^7/ml. No significant difference ($P > 0.05$) in the multiplication of each clone of *T. gallinae* was detected in HF medium with and without agar. The obtained values are much higher than those recorded by Honigberg (1961), who obtained 3.3×10^6/ml in simplified trypticase medium supplemented with agar.

For the growth of *T. gallinarum* another parameter needs special attention, the concentration of potassium phosphate. HF and TV media contain different amounts of monobasic and dibasic potassium phosphate. Altogether, the amount of potassium phosphate in all media tested other than Medium 199 is higher than in TV medium which could have an adverse effect on the growth of *T. gallinarum*. Consequently, the content of potassium phosphate was decreased by 50% to obtain modified TV medium. Improved growth was observed after this modification of the medium which makes it the first choice for propagation of *T. gallinarum*. This is further supported by the fact that there was no significant difference ($P > 0.05$) between the growth rates of both *T. gallinarum* clones investigated in TV medium or modified TV medium under different conditions.

The addition of serum has been considered essential for the growth of trichomonads since it provides lipids, fatty acids, amino acids and trace metals (Diamond, 1986). The results of this investigation showed that greater numbers of motile trophozoites were obtained in cultures containing 10% FCS in comparison to those containing a similar concentration of chicken serum. Obviously, some of the nutrients present in FCS have a favourable effect on the growth of both, *T. gallinarum* and *T. gallinae*.

So far there are no data available about aerobic and anaerobic growth conditions of *T. gallinarum* and *T. gallinae*. Although it is known that trichomonads are amitochondrial anaerobic protozoa, these flagellates are able to gain the required energy by utilizing exogenous and endogenous carbohydrates, under both aerobic and anaerobic conditions (Donald and Miklos, 1973). This function may confirm our results that *T. gallinarum* and *T. gallinae* were able to multiply under aerobic and anaerobic conditions following axenization.

Following axenization and optimization, no significant differences in the growth rate of different clones of *T. gallinarum* and *T. gallinae* in the presence or absence of antibiotics could be ascertained in different cultures. Therefore, the addition of antibiotics to the axenic cultures of *T. gallinarum* and *T. gallinae* had no adverse effect on the growth of these protozoa. Consequently, antibiotics could be added to the axenic cultures as a protective agent in order to prevent bacterial contamination.

Finally, we demonstrated that axenic cultures of *T. gallinarum* and *T. gallinae* were obtained by adopting a simple procedure avoiding the use of somewhat cumbersome V-tubes. In combination with micromanipulation, the described procedures are favourable to obtain axenic clonal cultures containing high yields of the respective protozoan parasite.

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