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Comparative study of five culture media for *in vitro* culture of *Trichomonas gallinae* from birds in Assam

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Abstract
A study was carried out in order to compare the *in vitro* efficacy of five different culture media against the flagellate protozoa, *Trichomonas gallinae*. For *in vitro* culture, oral swabs of *T. gallinae* were collected randomly from oropharynx and crop after visual inspection of birds as pigeon, duck, chicken and quail with a sterilized cotton swab and cultivated in five media namely modified Diamond media, Medium199, Minimum Essential Medium (MEM), RPMI 1640 and Nutrient broth. The initial inoculation of trophozoites into each media was $1X10^3$cell/ml. Among all the five media, Medium 199 showed the highest growth rate and motility of the organisms till 7 days after initial inoculation. All media were found suitable for harvesting and sub culture of *T. gallinae* under *in vitro* condition.

Keywords: *Trichomonas gallinae*, diamond media, medium199, minimum essential medium (MEM), RPMI 1640, nutrient broth

Introduction
Domestic birds like duck, chicken, geese, pigeon, quail, turkey etc. are more popular throughout the world. Poultry meat and eggs are among the most common animal protein source. Pigeon meat contains high protein and minerals that are beneficial for our body. Among the various types of parasites, *Trichomonas gallinae*, a flagellate protozoan, has significant health and economic impact on poultry industry, especially in pigeon and game birds rearing and breeding (Stockdale *et al*., 2015) [1] Typical lesions include development of caseous masses (canker) in the upper digestive tract which in severe cases become completely blocked and cause death by starvation (Mesa *et al*., 1961) [2]. Diagnosis of trichomoniasis is generally done by gross clinical symptoms and lesions exhibited by the affected birds and microscopic examination of fresh throat swabs/ oropharyngeal swabs collected from the birds. Cultivation of the protozoa *in vitro* in different media is also carried out widely for accurate diagnosis. *In vitro* cultivation of *T. gallinae* was considered as the gold standard for detection of trichomonads due to easy interpretation even in poorly infected birds (Stabler, 1954) [3]. The comparative evaluation of direct smear (Giemsa stain) and culture methods for detection of *Trichomonas gallinae* infection in pigeon and chicken of Assam was carried out by Saikia *et al*. (2022) [4] where it was concluded that culture of *T. gallinae* has been proved to be superior to wet mount preparation or staining methods in detection of the flagellate. Cell culture was adopted to differentiate between different strains of *T. gallinae* pathogenicity and it proved to be a sensitive tool (Honigberg *et al.,* 1964) [5] *T. gallinae* could be cultured in a variety of media including liquid, semi liquid types and semi-solid CPLM-agar medium. In the present study, five media were prepared in the laboratory for *in vitro* culture of *T. gallinae* and their comparative assessment was made based on parasite growth and motility upto 7 days of study.

Materials and methods
The aim of the study was to assess the efficacy of five culture media by *in vitro* culture and growth of *T. gallinae* obtained from oral swabs of domestic birds.

Preparation of Modified Diamond's Media

The Modified Diamond's media was prepared according to Diamond (1957) [6], adding ingredients like 20.0 g of trypticase, 10.0 g of yeast extract, 5.0 g of maltose, 1.0 g of L-cysteine hydrochloride, 0.2 g of ascorbic acid. Solution was brought up to 1,000 ml with distilled water and autoclaved for 15 min at 121 °C under 15 lb/in pressure for sterilization. Antibiotic mixture (Sodium penicillin G100000IU/100ml, Streptomycin sulfate 0.1 gm/100 ml) and 10% fresh inactivated fetal calf serum (inactivated by boiling at 56 °C for 30 minutes in water bath) were added to the solution. The pH of the culture media was adjusted to 6.5 with hydrochloric acid (Kharofa, 1999) [7].

Commercially available media

In the present study, the four commercially available media were purchased, i.e. Nutrient broth from HiMedia Laboratories, Mumbai, India; Medium 199 from Sigma Aldrich; Minimum Essential Medium (MEM) from Thermo Fisher Scientific, Invitrogen Bioservices India Pvt. Ltd. and Roswell Park Memorial Institute-1640 (RPMI-1640) from Thermo Fisher Scientific. Antibiotic mixture prepared for Modified Diamond's medium at same concentration was added to each media.

Counting of *Trichomonas gallinae*

For *in vitro* culture, oral swabs were taken from oropharynx and crop after visual inspection of birds as pigeon, duck, chicken and quail with a sterilized cotton swab. For each bird, swab was inoculated individually into tubes containing *Trichomonas* selective culture medium i.e. Medium 199. The initial inoculation in the media was 1×10^3 cell/ml (estimation of the number of trophozoites in culture was made in Neubauer chamber and counted at 400X magnification). Only motile *T. gallinae* organisms were counted as high (+++), moderate (++), less (+), weakly motile (+/-) and dead (-). After *in vitro* culture of the parasites, the concentration of the organisms increased many fold along with their motility and it became difficult to make live count, so Trypan blue (0.3%) dye was used to charge into the chamber for detection of parasites as the dead parasites took blue colour and it was easy to count the parasites. Firstly, the cultured samples were diluted in Trypan Blue dye by preparing a 1:1 dilution of the cell suspension using 0.3% solution of Trypan Blue. After adding the stain solution, the trophozoites of *T. gallinae* could be easily differentiated within a minute.

The cultured tubes were incubated at 37 °C in BOD incubator for seven days in aerobic condition for giving the protozoan parasites sufficient time to multiply. Culture tubes were monitored every 24 hours post inoculation for 7 days for assessment of growth and motility of the parasites and were kept tightly capped after sampling. Positive culture materials were transferred into new fresh medium with antibiotics and passages continued every 48-72 hours till bacterial contamination was controlled.

Results and discussion

The randomly collected throat swab samples of *Trichomonas gallinae* was cultivated in five media namely modified Diamond media, Medium199, Minimum Essential Medium (MEM), RPMI 1640 and Nutrient broth. The initial inoculation of trophozoites into each media was $1X10^3$cell/ml. The comparative evaluation on the performance of five media used for cultivation of *T. gallinae* is shown in Table 1 and Fig. 1.

Table 1: Comparative performance of culture media in relation to *in vitro* growth and motility of *T. gallinae*

Media 5 ml	Initial concentration of parasite/ml	Growth and motility of *T. gallinae*							
		24 hours		48 -72 hours		96-120 hours		144 -168 hours	
		Motility	Conc. of parasite/ml	Motility	Conc. of parasite/ml	Motility	Conc. of parasite/ml	Motility	Conc. of parasite/ml
Modified Diamond's medium	1000	++	2200	+++	4400	+	500	-	0
Nutrient broth	1000	++	1200	+	67	-	0	-	0
Medium 199	1000	+++	8200	+++	16000	++	10000	+	3700
MEM	1000	+	2300	++	3800	+	650	+/-	300
RPMI 1640	1000	+	2024	++	2600	+	752	-	0

N.B. +++= High ++= Moderate +=Less +/- = Weakly motile - =No motility/dead

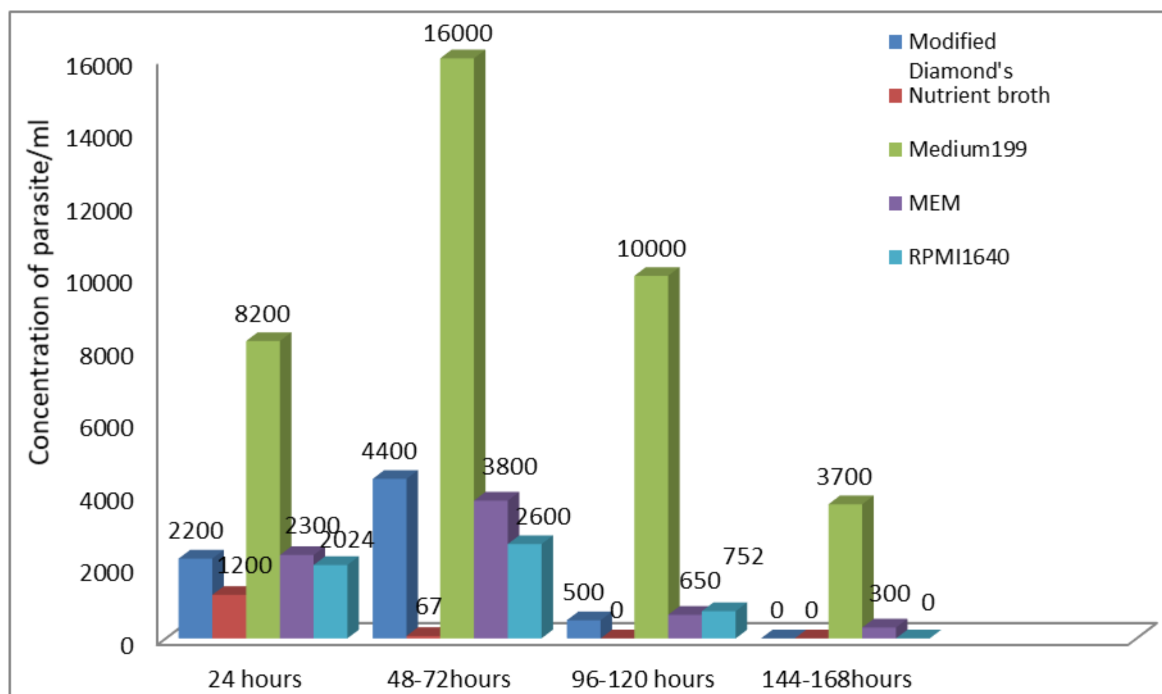

Fig 1: Comparative study on the performance of five media used for cultivation of *T. gallinae*

In modified Diamond's media, the activity of the *Trichomonas gallinae* organisms after 24 hours of incubation was moderate (++) with concentration of 2200 cells/ml with gradual increase of growth, reaching highest at 48 hours with concentration of 4400 cell/ml. Highest motility (+++) of the parasites was seen between 48-72 hours after initial inoculation. Gradually, from 96 hours onwards the motility of the organisms decreased (+) and their live count recorded was 500 cell/ml and within 144-168 hours (6-7 days) of initial inoculation of culture tubes, no live parasites were found. Modified Diamond's media was successfully used for the cultivation of *T. gallinae* by Diamond (1954) [8], Sansano Maestre *et al.* (2009) [9] and Amin *et al.* (2010) [10]. This rich liquid medium favored the multiplication of the parasite with an average incubation time of 48 hours for the detection of growth. However, some isolates needed a prolonged period of 7-10 days in order to detect active trophozoite movement. This feature was detected mainly in culture from necrophagous birds of prey that also demonstrated a smaller cell size during the examination of optical microscopy and later by morphometric analysis.

In Medium 199, same concentration of organisms (1×10^3 cells/ml) was inoculated and counting was carried out till 7 days. The trophozoites were highly motile (+++) and their numbers increased many folds to 8200 cell/ml after 24 hours of incubation of culture tubes at 37 °C. Their growth increased further reaching 16,000 cell/ml along with high motility (+++) 72 hours after initial inoculation when counted in haemocytometer. Even after 120 hours of incubation at 37 °C, the concentration of *T. gallinae* organisms was maintained up to 10,000 with moderately motile (++) trophozoites. Medium 199 could support the growth till 7th day of inoculation reaching 3700 cell/ml with gradual decrease of motility (+). This media gave the highest growth of organism in comparison with other media. Amin *et al.* (2010) [10] reported that viable trophozoites of *T. gallinae* 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 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. Use of antibiotics had no influence on the growth of *T. gallinae*.

Minimum Essential medium (MEM) gave the greatest growth between 48-72 hours of incubation, reaching up to 3800 cell/ml with moderately motile (++) trophozoites after $1\text{x}10^3$ cell/ml of initial inoculation. From 96-120 hours onwards, the number of growing trophozoites gradually decreased to about 650 cell/ml with less motility (+). Within 144-168 hours of inoculation, the organisms exhibited very weak motility and the number of cell was 300 cell/ml reaching nearly death stage.

In RPMI 1640 medium, the concentration of live organisms reached up to 2024 cells/ml after 24 hours of incubation of culture tubes at 37 °C with less motility (+). However, the highest growth of trophozoites was observed within 48-72 hours of incubation reaching a concentration of 2600cell/ml with moderately motile (++) organisms. Thereafter, the number of growing cells decreased gradually and the concentration was 752 cell/ml after 120 hours with extremely less motility. The parasites did not survive beyond 6 days and no live trophozoites were observed within this time.

In Nutrient broth, the survivality and concentration of the trophozoites of *T. gallinae* after initial inoculation of $1\text{x}10^3$cell/ml was highest after 24 hours reaching up to 1200 cell/ml with moderate (++) motility, then the number of living trophozoites decreased gradually after 24 hours. At the end of 72 hours only about 67 live organisms could be counted with less motility (+) and reaching the death stage.

Among all the five media, Medium 199 showed the highest growth rate and motility of the organisms till 7 days after initial inoculation. Moreover, all media were found suitable for harvesting and sub culture of *T. gallinae* under *in vitro* condition.

Hamad *et al.* (2017) [11] cultivated *T. gallinae* from domestic pigeon in Iraq in four culture media with initial inoculation of 10^5 cell/ml consisting of modified Diamond media (TYM), Cysteine Peptone Maltose media (CPLM), Trichomonas

CM0161 media (CM 161) and In-pouch media. The best growth of *T. gallinae* was observed in In-pouch media, Modified Diamond media and CM 161 giving high growth at 48 hours while CPLM gave high growth from 24 hours while CM 161 media showed acute decrease in the number of *T. gallinae* after 48 hour. In the same way, McLoughlin (1965) [12] used Diamond's medium for inoculation of material swabbed from the mouth and oesophagus of naturally infected and experimentally induced pigeon and incubated the cultures at 35˚C and examined for trichomonads after 48, 72 and 144 hours intervals and observed highest growth up to 72 hours thus conforming to our findings. In contrast to our report, Raza *et al.* (2018) [13] used two commercially available cultured media, modified Diamond's medium and modified thioglycolate medium to identify the growth of *T. gallinae* from oropharyngeal swabs of domestic pigeons in Lahore, Pakistan by incubating up to 72 hours at various temperatures and found modified thioglycolate medium practically efficient than Diamond's medium in recovering the parasites from specimens. Clonal cultures of trichomonads grown in Medium 199 developed high population in the presence of bacteria (Hess *et al.,* 2006) [14]. According to Grabensteiner *et al.* (2010) [15], Medium 199, a general maintenance media of eukaryotic cells, supplemented with rice starch and inactivated fetal bovine serum was a standard media for isolation of *T. gallinae* as was also seen to be the best media in our present study. Amin *et al.* (2010) [10] opined that semisolid media like Hollander fluid containing microbiology agar –agar was another media for cultivation of *T. gallinae*.

Nutrient Broth, MEM and RPMI 1640 have been used for the first time in cultivation of *T. gallinae* in India. The present study showed MEM and RPMI 1640 were not as efficient as Medium199 which might probably due to being formulated with sodium bicarbonate which play a role in maintaining pH of the culture medium. In the present study, Nutrient broth medium was found to be efficient in recovering the parasite from specimens and may provide a readily available, low cost substitute for the other media, while transporting throat swabs from the field to the laboratory.

Various factors influence the growth of flagellates *in vitro* such as media supplements that boost *T. gallinae*, cultivation method and an incubation temperature of 37 °C where number of live cells recorded was higher compared to that of culture at 40 °C (Tasca and De Carli, 2003 [16]; Amin *et al.,* 2010) [10] [16, 10] thus agreeing to our present study where cultivation of organism was done at 37 °C in aerobic condition. Additionally, antibiotics was added to all five media and this agrees to Amin *et al.* (2010) [10] who reported no influence of antibiotic on the growth of *T. gallinae* but anaerobic incubation was detrimental to their growth. However, Stabler *et al.* (1964) [17] speculated that adding of antibiotic in culture medium might lead to attenuation of organisms.

Conclusion

From the present work it can be concluded that all the five culture media were found suitable for harvesting and sub culture of *T. gallinae* under *in vitro* condition and Medium 199 proved to be the best media for culture. This work on comparative study is the first report from this part of the country

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