Veterinary Parasitology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Trichomonas gallinae, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures

Aziza Amin, Ivana Bilic, Evelyn Berger, Michael Hess*

Clinic for Avian, Reptile and Fish Medicine, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria

ARTICLE INFO

Article history:
Received 22 July 2011
Received in revised form 3 November 2011
Accepted 14 November 2011

Keywords:
Trichomonas gallinae
Tetratrichomonas gallinarum
LMH cells
QT35 cells
Cytopathogenic effect

ABSTRACT

In the present study the interaction of three genetically different clonal cultures of *Trichomonas gallinae* and *Tetratrichomonas gallinarum* with a permanent chicken liver (LMH) and a permanent quail fibroblast (QT35) cell culture was studied. Proliferation of *T. gallinae* cells was associated with a disintegration of the cell monolayer. The initial lesions on the LMH monolayer consisted of a progressive accumulation of the flagellate, forming clumps attached to the monolayer. A prolonged incubation time was characterized by appearance of holes in the cell monolayer with accumulation of trichomonads at their periphery. According to the severeness of the monolayer disruption differences among three tested *T. gallinae* clones were noticed. Furthermore, filtrates obtained either from axenic cultures of *T. gallinae* or from infected cell cultures produced a cytopathogenic effect similar to the protozoal cells, on both types of cell cultures. However, the destructive effect of the flagellates and their cell-free filtrates was much more pronounced on the LMH monolayer in comparison with the QT35 cells. Furthermore, freshly seeded LMH and QT35 cells suspended in cell-free filtrates of *T. gallinae* were unable to form a confluent monolayer. In comparison to *T. gallinae*, clonal cultures of *T. gallinarum* or their cell-free filtrates produced no effect on both types of monolayers. Interestingly, the cell-free filtrates obtained from both trichomonad species had an effect on the viability of both cell cultures. However, the cytotoxic effect of *T. gallinarum* filtrates was less severe than that recorded by *T. gallinae*. Consequently, for the first time a destruction of specified monolayers induced by *T. gallinae*-free filtrates could be demonstrated.

1. Introduction

Trichomonas gallinae is a known pathogen causing avian trichomonosis in various bird species, especially pigeons and doves. The flagellate commonly inhabits the upper part of the digestive tract (Stabler, 1954). Recently, several severe outbreaks were reported among wild birds due to *T. gallinae* infection resulting in a decreased number of these birds (Work and Hale, 1996; Neimanis et al., 2010; Robinson et al., 2010). Former studies have demonstrated that certain strains of *T. gallinae* vary with regard to their pathogenicity, ranging from non-pathogenic to highly pathogenic (Stabler, 1954; Bondurant and Honigberg, 1994). Interestingly, prolonged axenic cultivation of a very virulent *T. gallinae* strain (Jone's Barn (JB) strain) in non-living media caused a loss in pathogenicity (Goldman and Honigberg, 1968). Differences in pathogenicity may also reflect the genetic diversity reported for *T. gallinae* strains (Gerhold et al., 2008; Anderson et al., 2009; Grabensteiner et al., 2010).

In vitro systems based on tissue cultures are a suitable method for studying a cytopathogenic effect of protozoa

* Corresponding author. Tel.: +43 1 25077 5151; fax: +43 1 25077 5192.
E-mail address: michael.hess@vetmeduni.ac.at (M. Hess).

0304-4017/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2011.11.037

(Alderete and Pearlman, 1984; Garber et al., 1989), because the direct observation of monolayer destruction enables an efficient determination of the mechanism of pathogenicity of a certain strain. However, only a few studies were performed so far demonstrating the infection of cell cultures with *T. gallinae* (Honigberg et al., 1964; Kulda, 1967). Honigberg et al. (1964) examined the effect of one virulent (Jone's Barn) and a non-virulent (Lahore) strain on a trypsin-dispersed chick liver cell culture. The authors showed that there are significant differences between the two strains, while the effect of a cell-free filtrate obtained from an actively grown virulent trichomonad strain was relatively minor. In a second study a monkey kidney cell line was used as substrate (Kulda, 1967), suggesting that abnormal changes in the cell culture were induced by *T. gallinae* but not by the supernatant of the growth media. In addition to these studies, destruction of tissue culture cells through direct contact of protozoa is well described for *Trichomonas vaginalis*, a close relative of *T. gallinae* (Alderete and Pearlman, 1984; Arroyo and Alderete, 1989; Gilbert et al., 2000). Cytopathogenic changes in tissue culture were also observed following the exposure of cells to toxic substances released by *T. vaginalis* into the culture media (Garber et al., 1989; Pindak et al., 1993; Fiori et al., 1996; Sommer et al., 2005).

Tetratrichomonas gallinarum is a flagellated protozoan with a worldwide distribution, commonly inhabiting the intestinal tract of different poultry species (Friedhoff et al., 1991). Until now there are some controversies about the pathogenicity of *T. gallinarum* (Allen, 1941; Norton, 1997). This was also attributed to the frequent occurrence of mixed infections with other protists like *Histomonas meleagridis* and *Blastocystis* sp. (Tyzzer, 1920). Recently, we demonstrated the absence of clinical signs, macroscopic and microscopic lesions following infection of either turkeys or specified pathogen-free chickens with *T. gallinarum* (Amin et al., 2011). The lack of pathogenicity is in agreement with the study mentioned above in which *T. gallinarum* strains grew on monkey kidney cell culture without any effect on these cells (Kulda, 1967).

As none of the studies reported so far applied genetically defined clonal cultures to different kind of cells the present study had several aims. Firstly, to demonstrate the direct interaction of genetically diverse clonal cultures of *T. gallinae* and *T. gallinarum* with permanent chicken liver (LMH) and permanent chicken fibroblast (QT35) cells and secondly, to elucidate possible differences between trichomonads and their cell-free filtrates on these cells.

2. Materials and methods

2.1. Clonal cultures

Three genetically different clonal cultures of *T. gallinae* named *T. gallinae*/Budgerigar/Austria/5895-C1/06 (briefly, clone 5895-C1/06), *T. gallinae*/Racing Pigeon/Austria/8855-C3/06 (clone 8855-C3/06), *T. gallinae*/Racing Pigeon/Austria/231-C1/07 (clone 231-C1/07), and three genetically different clones of *T. gallinarum*: *T. gallinarum*/Turkey/Austria/2721-C7/03 (clone 2721-C7/03), *T. gallinarum*/Turkey/Germany/4114-C5/05 (clone 4114-C5/05), *T. gallinarum*/Turkey/Austria/8386-C15/06 (clone 8386-C15/06) were investigated (Grabensteiner et al., 2010; Amin et al., 2010). The assignment reflects the species of bird/country of origin/diagnostic number-clone number/year of isolation. All clonal cultures were investigated at two different *in vitro* passages (P) ranging from P10 to P121 for *T. gallinae* and P8–P575 for *T. gallinarum*. Exceptional to this, the *T. gallinarum* clonal culture 8386-C15/06 was investigated at passage level 8 and 12, only. High passages were achieved by routine transferring of cultures into fresh media every 48 h as described recently (Amin et al., 2010).

2.2. Cell cultures

Permanent chicken liver (LMH) and a Japanese quail fibroblast (QT35) cell lines were grown in Medium 199 (M199) + Earle's salts + L-glutamine and Dulbecco's minimal essential medium (DMEM) + D-glucose + L-glutamine + sodium phosphate + sodium pyruvate (Invitrogen/Gibco, Paisley, UK), respectively (Kawaguchi et al., 1987; Moscovici et al., 1977). Both media were supplemented with 10% of fetal bovine serum (FBS), 10% tryptose phosphate broth, 0.5% from a stock solution containing penicillin (40,000 IU/ml) and streptomycin (40 mg/ml) (all Invitrogen/Gibco, Paisley, UK). 1 ml of the media suspension containing 1×10^6 cells was inoculated into 75 cm^2 flasks with filtered caps (Sarstedt, Wiener Neudorf, Austria) containing 6 ml medium. Cells were kept in a controlled atmosphere of 5% CO_2 at 37 °C and around 85–90% humidity. Cells were only used if a confluent monolayer of LMH and QT35 cells was noticed which usually appeared after 72 h of incubation and 7×10^6 cells per flask were counted on average. Cells were passaged every three to four days depending on their density.

2.3. Propagation and preparation of trichomonad cultures for infection

Following axenization, *T. gallinae* clones were grown in Hollander fluid (HF) medium, while *T. gallinarum* clones were grown in modified *T. vaginalis* (TV) medium as described recently (Amin et al., 2010). At 24 h post incubation (p.i.), the trichomonad culture was centrifuged at $3300 \times g$ for 5 min; the pellet was washed three times by suspension in phosphate buffer saline (PBS, pH 7.2) and centrifugation. Afterwards the pellet was suspended in 1 ml M199 or DMEM for LMH and QT35 cells, respectively. Motile trichomonads were counted by a Neubauer cell counting chamber (Reichert, Buffalo, NY). The concentration of the inoculum was adjusted to contain approximately 10^3 and 10^5 motile trophozoites suspended in 500 μl medium. Confluent LMH and QT35 monolayers were infected with *T. gallinae* and *T. gallinarum* trophozoites. Cells were kept in a controlled atmosphere of 5% CO_2 at 37 °C and around 85–90% humidity and examined daily using a light inverted microscope, to assess the degree of monolayer destruction. Control flasks containing cell cultures only, or flasks with trichomonads suspended in relevant media without tissue culture cells were incubated under the same condition. To measure the effect of

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

different concentrations of individual trichomonads at different passages on intact cell cultures the experiments were done in duplicate and repeated twice. For calculation of the final score the mean of each treatment was used.

2.4. Growth of trichomonads in cell cultures

For proliferation assay each monolayer was infected with 10^5 trichomonads, which were counted at different time points (24, 48, 72, 96 and 120 h p.i.). For this, the medium from infected flasks was aspirated and the monolayer was washed two times with PBS which was later added to the medium. After this the monolayer was trypsinized with 0.2% trypsin to detach cells, and trichomonads were counted. The total number of trophozoites, consisting of trichomonads which were not attached to the cells and present in the mixture of medium and PBS together with the trophozoites attached to the cell cultures, was determined. To evaluate if both cell lines secreted a growth factor able to sustain the growth of trichomonads, 10^5 protozoal cells were suspended in filtrates obtained from supernatants collected from non-infected LMH and QT35 monolayers. Such filtrates were collected at different time points (24, 48, 72 h p.i.). This growth assay was also performed by inoculating 10^5 trichomonads in fresh M199 and DMEM. Each treatment was done in triplicate and the mean of each treatment was used for calculating the number of viable flagellates.

2.5. Preparation of cell-free filtrates either from axenically grown trichomonad cultures or from infected LMH and QT35 cells

For preparation of cell-free filtrates different concentrations (10^3, 10^5, and 10^7 cells) of *T. gallinae* and *T. gallinarum* cultures were incubated for 24 h in M199 or in DMEM, the same medium as used for growing LMH and QT35 cells, respectively. Prior to incubation the protozoal cells were washed three times with PBS (pH 7.2). For this, trichomonad cultures were centrifuged at 3300 × *g* for 5 min after which the supernatants from the cultures were filtered through a 0.22 μm cellulose acetate filter (Millipore, VWR) as described for *T. vaginalis* (Garber et al., 1989). Cell-free filtrates were also prepared from dead flagellates which were obtained from tubes incubated for 72 h.

Additionally, cell-free filtrates were collected at different time intervals (24, 48 and 72 h p.i.) from infected LMH or QT35 monolayers and prepared as mentioned above. Absence of trichomonads was confirmed by incubation of the cell free-filtrate in flasks without cell culture throughout the whole incubation period.

The pH of the filtrates was adjusted to the pH of the fresh medium used for growing the cell cultures. The filtrates were added to LMH or QT35 cells and incubated in 5% CO_2 at 37 °C and around 85–90% humidity. The filtrates were tested in duplicate with two repetitions and the mean of each treatment was used for evaluation of the scoring lesion produced by cell-free filtrates in cell cultures.

Furthermore, all filtrates were also added to freshly seeded LMH and QT35 cells to investigate the effect of these filtrates on the proliferation rate of such cells. Each treatment was done in duplicate.

2.6. Assessment of LMH and QT35 monolayers

Each monolayer was investigated visually by an inverted light microscope (10×) to detect the effect of the protozoal cells and the cell-free filtrates on LMH and QT35 monolayers. According to the degree of monolayer destruction, the following scoring system was established:

0 = intact monolayer
1 = up to 25% of the monolayer destructed
2 = 25–50% of the monolayer destructed
3 = 50–75% of the monolayer destructed
4 = more than 75% of the monolayer destructed.

2.7. Determining the effect of axenically derived cell-free filtrates on the viability of LMH and QT35 cells

Viability of LMH and QT35 cells was determined by trypan blue staining following trypsinization of the monolayer. This procedure was carried out following incubation of the cell cultures with filtrates collected from 10^7 axenically grown trichomonad-cultures after 24 h of incubation as described above. The cells were counted using a counting chamber (Reichert, Buffalo, NY) and cell viability was determined as a percentage of the total cells counted. Viability of the cell cultures was determined at different time points (24, 48 and 72 h p.i.). Moreover, the ability of these cells to form a monolayer after washing, trypsinization and suspension in fresh medium was investigated, as additional criteria for viability. Each sample was done in triplicate and the mean of these counts was used for calculation of the percentage of the viability of LMH and QT35 cells.

2.8. Cytotoxicity of cell-free filtrates obtained from axenically grown trichomonad cultures

To confirm the effect of the cell-free filtrates on the viability of cell cultures, CellTiter 96® aqueous one solution cell proliferation assay (Promega Corporation, Madison, USA) was used following manufacturer's instructions. The cell-free filtrates from 10^7 axenically grown trichomonad cells were prepared as described above. A total of 200 μl of the filtrates was added to confluent LMH and QT35 monolayers grown in 96-well plates. The viability of the cells was demonstrated at different time points (24, 48 and 72 h p.i.). For control, the monolayers were incubated with fresh medium only. Each sample was done in quadruplicate and the data was expressed as mean ± MSD. The absorbance values were recorded at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. The cytotoxicity in this assay was defined as $1 - E/C$, where E is the absorbance values of experimental samples and C is the absorbance value of control samples as described previously for *T. vaginalis* (Gilbert et al., 2000). The obtained values were recorded as percentage values and the statistical analysis was done by ANOVA followed by Tukey's test. P values of ≤ 0.05 were considered significant.

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

2.9. The effect of different pH values on LMH and QT35 monolayers

To assess the effect of different pH values on LMH and QT35 monolayers, 1 M NaOH and 1 M HCl were used to adjust the pH of the media to various degrees (6, 6.5, 7, 7.5, 8 and 8.5). Unadjusted media were used as controls. Beside visual examination of the monolayer by an inverted microscope, the viability of the cells incubated with different pH values at various time points (24, 48, 72, 96, 120 and 144 h p.i.) was determined by both trypan blue staining and CellTiter 96® aqueous one solution cell proliferation assay (Promega Corporation, Madison, USA). The experiments were done in triplicate and the mean of each treatment was used for calculation the percentage of cell cultures viability.

3. Results

3.1. Growth of trichomonads in cell cultures

After inoculation of LMH and QT35 monolayers with 10^5 *T. gallinae* cells which underwent only a few *in vitro* passages, the number of trophozoites of different *T. gallinae* clones increased (Fig. 1A and C). In the absence of either LMH or QT35 cells the protozoa were still able to survive for at least 24 h in the respective tissue culture media, although multiplication rate was rather low (Fig. 1A and C). The time of survival could be extended if the protozoa were incubated together with the filtered supernatant obtained from non-infected LMH or QT35 cells at various time points (24, 48 and 72 h p.i.). Exemplarily, the growth of protozoa in supernatants from LMH and QT35 monolayers collected after 72 h of incubation is shown in Fig. 1A and C. No significant differences ($P > 0.05$) in the proliferation rate could be demonstrated between different clonal cultures of *T. gallinae* on LMH and QT35 cells. In comparison to *T. gallinae,* the presence of LMH or QT35 cells had no significant effect on the growth of *T. gallinarum* (Fig. 1B and D). The number of *in vitro* passages had no effect on the proliferation of all tested *T. gallinae* and *T. gallinarum* cultures.

3.2. Interaction of trichomonads with cell cultures

The interaction of three *T. gallinae* clones applying different concentrations (10^3–10^5) with LMH cells was analyzed from 24 h until 120 h post infection (Fig. 2). After 24 h post infection the inoculated organism tended to attach to the monolayer and proliferate, forming clumps of the protozoan cells (Fig. 2 B). The size and number of such clumps increased with prolonged incubation time and destruction of the monolayer firstly occurred beneath the clumped trichomonads. Focal areas free from cell culture appeared, with aggregation of protozoon at the periphery of these area (Fig. 2C). These area expanded gradually (Fig. 2D–F). Complete detachment of the LMH monolayer (score 4) was caused by *T. gallinae* 5895-C1/06 at passage number 10 (P10) and clone 231-C1/07 P16 at different time points of incubation (Fig. 3), where motile trophozoites were noticed in the supernatant clustering around the detached cells. The third *T. gallinae* clone, 8855-C3/06 P16 caused destruction of about 50% of the monolayer (score 2) (Fig. 3 A). A similar degree of monolayer destruction could be induced by infecting LMH cells with 10^3 protozoal cells of the same passage level, even though an extended incubation time was needed (Fig. 3B). Similar results were noticed using the same amount of protozoa for infection obtained from prolonged *in vitro* passages such as P115 for clone 5895-C1/06 and P75 for both, clones 231-C1/07 and 8855-C3/06. Again, a somewhat longer incubation period was needed to observe the same effect in comparison to those cultures which underwent only a few *in vitro* passages (Fig. 3A and B).

The effect of all investigated *T. gallinae* clones on QT35 monolayer was a much less pronounced than the one recorded on LMH cells. Hence, the initial inoculum of 10^5 cells of *T. gallinae* clones 5895-C1/06 P10 and 231-C1/07 at P16 and P95 produced destruction of about 50% of the QT35 monolayer (score 2). The third clonal culture named *T. gallinae* 8855-C3/06 at both passage levels P16 and P95 as well as the clonal culture 5895-C1/06 P135 destroyed only 25% of the QT35 monolayer (score 1). A maximum lesion score of 1 was achieved by infecting the QT35 monolayer with 10^3 cells from different *T. gallinae* cultures.

All cultures of *T. gallinarum* (2721-C7/03 at P238 and P435, 4114-C5/05 at P8 and P75 and 8386-C15/06 at P8) showed no distinct effect on both LMH and QT35 cells, independent of the concentration of the inoculums and the passage numbers. In this case the trichomonad cells appeared individually swimming in the medium, and did not adhere to the monolayers.

3.3. Effect of cell-free filtrates on cell cultures

No growth of trichomonads was noticed in flasks containing cell-free filtrates only, in the presence or absence of cell cultures. The effect of cell-free filtrates collected after 24 h of incubation from axenically grown 10^7 *T. gallinae* cells on the LMH monolayer was similar to the effect described above (Section 3.2), when cells were directly infected with the protozoa (Fig. 3C). The severity of effects varied between different cell-free filtrates harvested from individual *T. gallinae* cultures. Entire detachment of the monolayer was noticed at 96 h p.i. with filtrate of clone 5895-C1/06 P16 (Fig. 3 C). At the same time the high passage number (P121) of this clone produced a reduced destruction of 75% (score 3) (Fig. 3C). In contrary, the filtrates obtained from 10^7 dead trichomonad cells had no adverse effect on the monolayer even at 144 h p.i. Additionally, the effect of the filtrates obtained from axenically grown 10^3 and 10^5 trichomonads after 24 h of incubation was analyzed. In this case the monolayer remained intact for 144 h p.i. Moreover, filtrates from a LMH cell culture infected with 10^5 *T. gallinae* cells were collected at different time points (24, 48 and 72 h) post infection. The filtrate obtained at 72 h post infection showed the strongest destructive effect on the monolayer (Fig. 3D) in comparison to those harvested at 24 and 48 h (data not shown).

Filtrates obtained from 107 T. gallinae cells, following either axenic growth for 24 h or incubated on QT35 cell culture, prodcued a much less destructive effect on the QT35 monolayer in comparison to LMH cells (data not

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

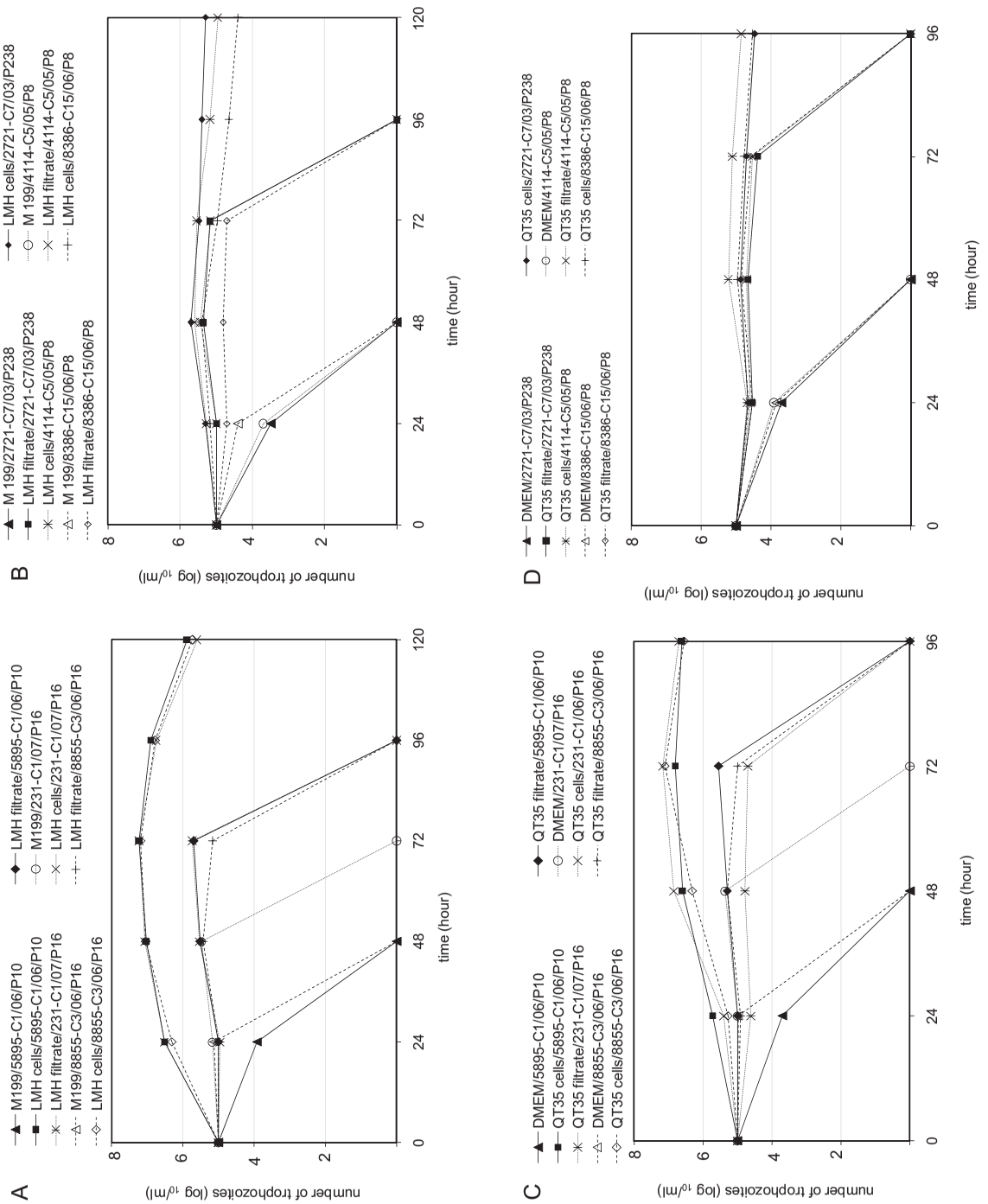

Fig. 1. Growth of different trichomonads in medium, or in the supernatant filtrate collected at 72 h p.i. from an non-infected monolayer. Results are displayed differently for LMH cells: (A) *T. gallinae*, (B) *T. gallinarum* and QT35 cells, (C) *T. gallinae*, (D) *T. gallinarum*.

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

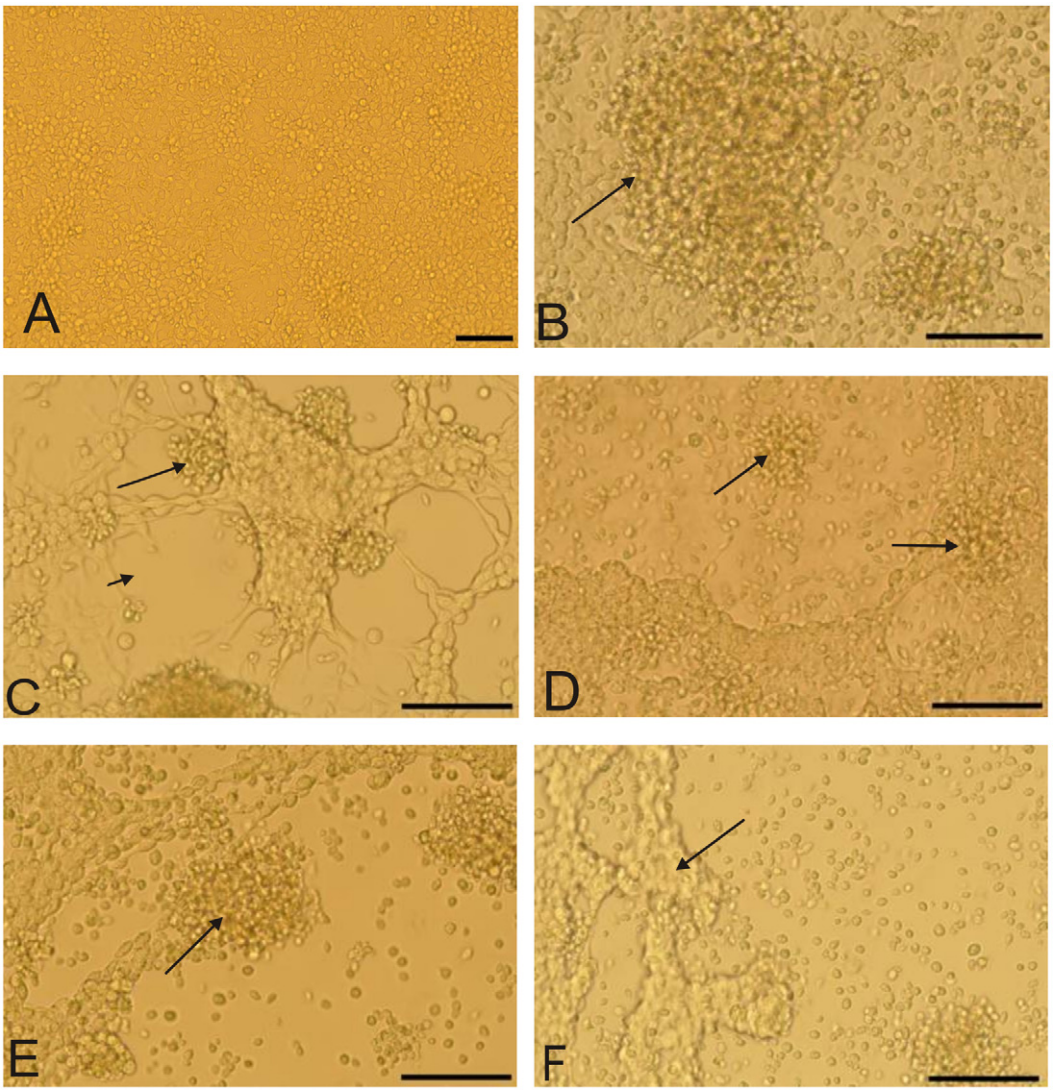

Fig. 2. Cytopathogenic effect in LMH monolayer inoculated with *T. gallinae* clone 5895-C1/06 P10: (A) non-infected LMH monolayer, (B) formation of clumps of trichomonads with an intact monolayer, (C) first focal area free from LMH cells (short arrow) with accumulation of the protozoa at the periphery of these area (long arrow), (D) increase in size of the destructed area with aggregation of trichomonads at the periphery or in the center of these area (arrow), (E) extended area with masses of accumulated trichomonads (arrow), (F) masses of floating trichomonads with remnant of monolayer cells (arrow). For photography live unstained cell culture was recorded (Bar = 20 μm).

shown). The most severe destruction of the QT35 monolayer was 50% (score 2) following incubation with cell-free filtrates from axenically grown clones 5895-C1/06 P10 and 231-C1/07 P16. Filtrates, obtained either from infected monolayers with different clonal cultures of *T. gallinarum* at various time points (24, 48 and 72 h) post infection or those from 10^7 axenically grown cultures for 24 h, had no effect on LMH and QT35 cells.

Different pH values of the medium (6, 6.5, 7, 7.5, 8 and 8.5) had no destructive effect on the confluence of the LMH monolayer. The QT35 monolayer remained intact during incubation with the adjusted medium (7, 7.5, 8 and 8.5), but destruction occurred at pH 6 and 6.5 after 24 h and 48 h of incubation, respectively. In all experiments the control flasks, including the monolayer incubated with fresh medium only, remained intact throughout the entire period of examination.

3.4. Effect of cell-free filtrates on freshly seeded LMH and QT35 cells

Filtrates obtained from LMH and QT35 cells 72 h post infection with *T. gallinae* 5895-C1/06 and 231-C1/07, at both low and high passage levels, inhibited the proliferation of LMH and QT35 cells. Consequently, freshly seeded LMH and QT35 cells suspended in these filtrates failed to form a confluent monolayer even at 144 h p.i. Similar results were demonstrated after suspending of LMH and QT35 cells in cell-free filtrates collected after 24 h of axenic growth of *T. gallinae* (10^7 cells). In contrast to this, freshly

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

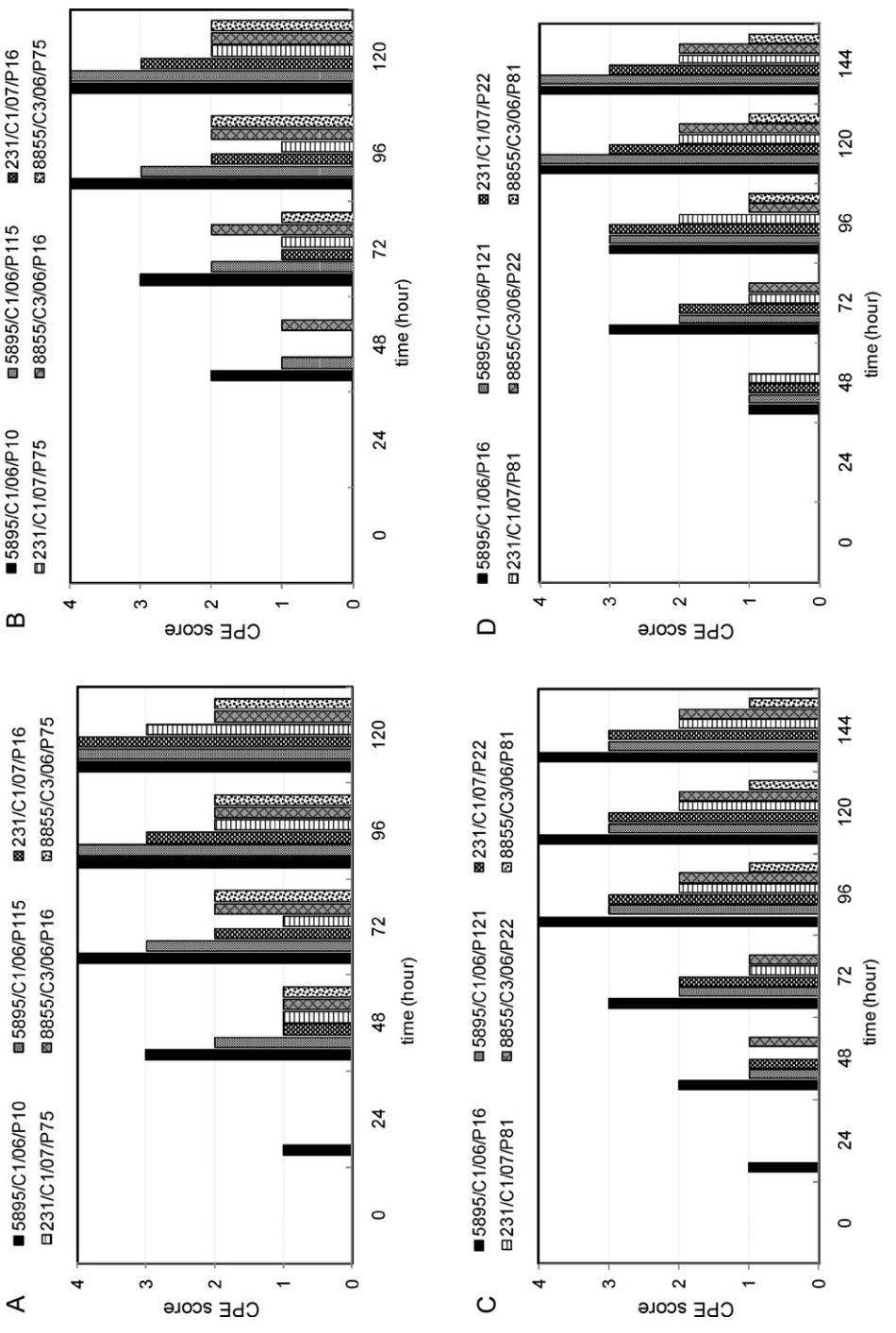

Fig. 3. Mean CPE scores of LMH monolayer incubated with genetically different *T. gallinae* clones following low and high *in vitro* passages at different time points: (A) 10^5 protozoa, (B) 10^3 protozoa or with cell free filtrates, (C) obtained after 24 h of incubation of 10^7 axenically grown protozoa, (D) filtrates harvested at 72 h post infection from LMH cell culture infected with 10^5 trichomonads.

seeded LMH and QT35 cells suspended in the filtrates of *T. gallinae* 8855-C3/06, independent of the *in vitro* passage, harvested from infected tissue cultures or from axenically grown culture, were able to form a confluent monolayer at 120 h p.i.

Cell-free filtrates obtained either from infected LMH and QT35 monolayers at 72 h post infection or from 10^7 axenically grown cultures of *T. gallinarum* had no inhibitory effect on the proliferation rate of LMH and QT35 cells. The control experiment was performed with the filtrate obtained from non-infected tissue cultures at 72 h p.i. which supported the formation of both LMH and QT35 monolayers at 72 h post seeding of cells.

3.5. Effect of cell-free filtrates on the viability of cell cultures

The viability of LMH and QT35 cells was first assessed by trypan blue staining. The cell-free filtrates of *T. gallinae* had an effect on the viability of the LMH and QT35 cells which decreased with the length of incubation (Fig. 4A and B). These results were confirmed by the cytotoxicity assay measuring viability with a CellTiter 96® aqueous one solution. An increase in cytotoxicity in association with the length of incubation could be demonstrated (Fig. 4C and D). No significant difference ($P > 0.05$) was obtained between genetically different *T. gallinae*-free filtrates applying both, trypan blue staining and CellTiter 96® aqueous one solution cell proliferation assay, as shown in Fig. 4.

T. gallinarum-free filtrates demonstrated slightly lower effects in cell cultures viability in comparison to *T. gallinae*-free filtrates (Fig. 4A and B). These results were confirmed with the cytotoxicity assay as shown in Fig. 4C and D. In all experiments the remaining cells were able to proliferate and form a confluent monolayer.

No significant effect ($P > 0.05$) on viability of LMH cells, determined by trypan blue staining and CellTiter 96® aqueous one solution cell proliferation analyses, was noticed following adjustment of fresh media to different pH values (6.5, 7, 7.5, 8 and 8.5). The same results were obtained for QT35 cells at pH values of 7, 7.5, 8 and 8.5. However, pH values of 6 for LMH cells together with 6 and 6.5 for QT35 cells had a significant negative effect ($P < 0.05$) on viability of these cells.

4. Discussion and conclusion

In the present study, the effects of genetically different clonal cultures of *T. gallinae* and *T. gallinarum* or their cell-free filtrates were investigated on two kinds of cell cultures. Because the liver is one of the predilection sites for *T. gallinae in vivo* (Narcisi et al., 1991), the LMH cell culture would provide a suitable model for studying this interaction *in vitro*. The QT35 cells were used because *T. gallinae* is well known to induce severe lesions in the epithelium of the pharynx. Accordingly, differences between various cell types could be addressed.

The present study showed that trichomonads were able to multiply on LMH or QT35 cells which required attachment of the parasites to these cells. Obviously, cell cultures acted as a source for nutrients since the cell-free supernatant from non-infected cell cultures as well as the media used for propagating cell cultures alone did not support the growth of the protozoan cells. A similar observation on McCoy cells was made by Karen et al. (1990) following infection with *T. vaginalis*, a close relative to *T. gallinae*. This indicated that eukaryotic cell membrane components may be important in supporting the growth of trichomonads, since it consists of a lipid bilayer with intrinsic and extrinsic proteins (Karen et al., 1990). This assumption is in agreement to the finding of Peterson and Alderete (1984) who demonstrated that lipoproteins are capable of supporting parasite multiplication. This correlated with the fact that *T. vaginalis* has specific receptors for lipoprotein binding and uptake on its surface (Peterson and Alderete, 1984). Interestingly, the present study demonstrated that *T. gallinarum* clones did not adhere to the monolayer which would explain why the protozoal cells did not grow in high numbers like *T. gallinae* clones.

Although, different clonal cultures of *T. gallinae* showed a nearly similar growth in cell cultures, variations in the degree of monolayer destruction were demonstrated, which probably reflect, differences in pathogenicity. A correlation between the degree of cell-damage caused by different strains of the same species and their inherent pathogenicity level was already described for *Tritrichomonas fetus* (Kulda and Honigberg, 1969). Furthermore, variations between different *T. gallinae* clonal cultures confirmed that the destructive effect of *T. gallinae* in cell cultures was not due to exhaustion of nutrients needed for the maintenance of the monolayer by flagellates.

Even though trichomonads proliferated in the presence of QT35 cells in the same manner as in LMH cells, their destructive effect on the QT35 monolayer was considerably lower. These variations may be due to differences in the sensitivities of the tissue cultures to *T. gallinae* clones or additional factors involved in the manifestation of cytopathogenic effect (CPE) in cell cultures (Gentry et al., 1985). This phenomenon is possibly related to the nature of the parasite–host cell interaction which may occur through specific receptor–ligand connections as described before for *T. vaginalis* by Alderete and Garza (1985). Moreover, this finding may also clarify the inability of *T. gallinarum* clones to adhere to LMH and QT35 cells and consequently explain the absence of a CPE in both cell lines. However, the results from the present study clearly demonstrated that the LMH monolayer is more tolerant for lower pH values than the QT35 cells. Therefore, LMH cells maintained for a longer period without spontaneous degeneration of the monolayer, which makes it more suitable to study the trichomonad–host interaction. These differences could be important during future studies aiming for recognition of cytopathogenic mechanisms of this parasite.

In the present study, different *T. gallinae* cultures produced a destructive effect on LMH and QT35 cells by both direct contact of the protozoa with the cell cultures and indirectly through interaction of molecules secreted during the protozoan growth. Both mechanisms explain the increased destruction of the monolayer even in the area without adherent protozoa, as described before for *T. vaginalis* (Silva-Filho and de Souza, 1988). The presence of viruses within the trichomonads may add up to the noticed

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

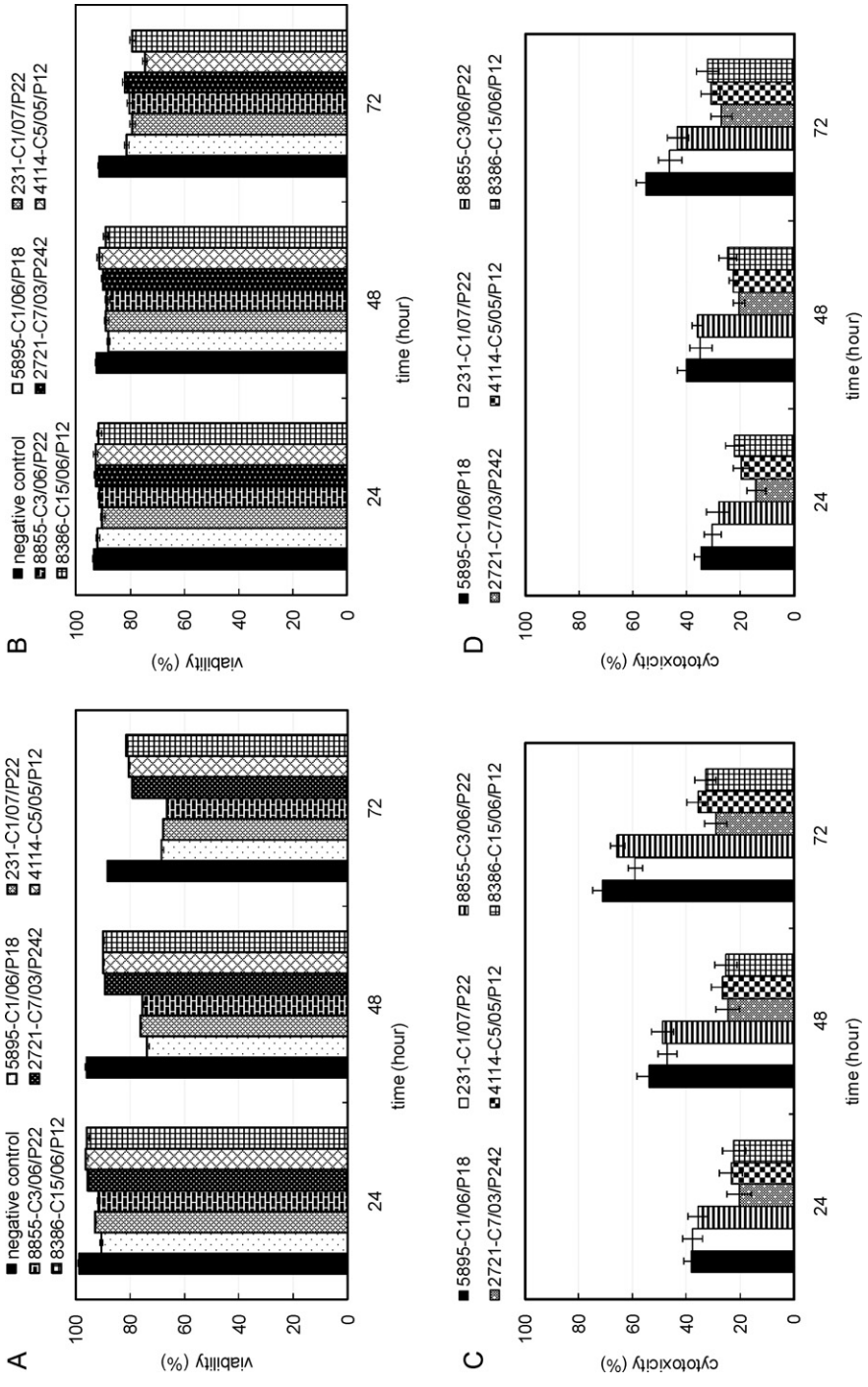

Fig. 4. Viability demonstrated by trypan blue staining of: (A) LMH cells, (B) QT35 cells, and cytotoxicity assessed by promega CellTiter 96® aqueous solution of (C) LMH cells and (D) QT35 cells. Absorbance values were recorded at 490 nm using ELISA reader after incubation with cell-free filtrates, obtained after 24 h of incubation of 10^7 axenically grown protozoa of different clonal cultures of *T. gallinae* and *T. gallinarum*, at different time points.

effect. A double-stranded RNA virus within the parasitic cell was reported for *T. vaginalis*, without knowing the effect this may have on tissue culture (Gerhold et al., 2009). However, previous investigation by electron microscopy of the most virulent strain used in the present study does not reveal a viral structure (Mehlhorn et al., 2009).

Cell-free filtrates were prepared to investigate the role of secreted products in trichomonad–cell interaction in detail. It could be assumed that variations observed with regard to monolayer destruction by different cell-free filtrates are attributed to the quantity and composition of the secreted molecules. The same interpretation could be made by comparing the CPE of cell-free filtrates obtained from low and high passage numbers of the same clonal culture. Data demonstrating the dependence of the CPE on the quantity of the initial inoculums in combination with the length of protozoan growth prior to collection of the filtrates support this assumption. Overall, these findings were similar to that demonstrated for *T. vaginalis* (Gentry et al., 1985; Garber et al., 1989). Further evidence for certain proteolytic activity present in cell-free filtrates came from the observation that freshly seeded LMH and QT35 cells do not form a monolayer when suspended in such filtrates.

In addition to the effects described above, cell-free filtrates of *T. gallinae* influenced the viability of LMH and QT35 cells. This is in agreement with an earlier observation reported by Garber et al. (1989), who investigated the effect of a cell detaching factor of *T. vaginalis* on tissue cultures. CellTiter 96® assay provided a quantitative colorimetric method to determine the viability of metabolically active cells. Variations in cytotoxicity among different trichomonad clones are most likely based on the differences in the amount of the secreted cytotoxic products by each individual clone, as reported for *T. vaginalis* (Krieger et al., 1985; Alderete et al., 1987). Moreover, in the present investigation it could be demonstrated that various pH values had no effect on the confluence of the LMH monolayer, which would confirm the presence of products secreted by trichomonads involved in monolayer destruction. Similar results were demonstrated on McCoy cells, as a pH less than 6 had no effect on monolayer confluence, but it had a direct effect in cell viability (Garber et al., 1989).

Interestingly, even though *T. gallinarum* filtrates did not produce any destructive effect on the monolayers, these cell-free filtrates had an effect on the viability of both cell lines but were insufficient to cause destruction of the monolayers. This could be explained not only by the nature of the secreted molecules, but also by their quantity, as mentioned for haemolytic pore-forming molecules produced by *T. vaginalis* (Fiori et al., 1996). Accordingly, molecular analysis of cell-free filtrates from *T. gallinarum* and *T. gallinae* would be needed to answer such questions. The established tissue culture systems could be a useful tool for further investigations, because both types of trichomonads behave differently, following infection of these cell lines.

In conclusion, the present study showed that genetically different *T. gallinae* clones or their filtrates caused severe destruction of specific cell cultures. Obviously, products secreted by this parasite possess proteolytic activity, which contributed to such an effect. The function and nature of these molecules noticed *in vitro* needs further clarification, which would be helpful to elucidate the mechanism of pathogenicity of *T. gallinae in vivo* and the trichomonad–host cell relationship in greater detail.

References

Alderete, J.F., Demes, P., Gombosova, A., Valent, M., Yanoska, A., Fabusova, H., Kasmala, L., Garza, G.E., Metcalfe, E.C., 1987. Phenotypes and protein–epitope phenotypic variation among fresh isolates of *Trichomonas vaginalis*. Infect. Immun. 55, 1037–1041.

Alderete, J.F., Garza, G.E., 1985. Specific nature of *Trichomonas vaginalis* parasitism of host cell surfaces. Infect. Immun. 50, 701–708.

Alderete, J.F., Pearlman, E., 1984. pathogenic *Trichomonas vaginalis* cytotoxicity to cell culture monolayers. Br. J. Vener. Dis. 60, 99–105.

Allen, A., 1941. Macroscopic differentiation of lesions of histomoniasis and trichomoniasis in turkeys. Am. J. Vet. Res. 2, 214–217.

Amin, A., Neubauer, C., Liebhart, D., Grabensteiner, E., Hess, M., 2010. Axenization and optimization of *in vitro* growth of clonal cultures of *Tetratrichomonas gallinarum* and *Trichomonas gallinae*. Exp. Parasitol. 124, 202–208.

Amin, A., Liebhart, D., Weissenböck, H., Hess, M., 2011. Experimental infection of turkeys and chickens with a clonal strain of *Tetratrichomonas gallinarum* induces a latent infection in the absence of clinical signs and lesions. J. Comp. Pathol. 144, 55–62.

Anderson, N.L., Grahn, R.A., Van Hoosear, K., Bondurant, R.H., 2009. Studies of trichomonad protozoa in free ranging songbirds: prevalence of *Trichomonas gallinae* in house finches (*Carpodacus mexicanus*) and corvids and a novel trichomonad in mockingbirds (*Mimus polyglottos*). Vet. Parasitol. 161, 178–186.

Arroyo, R., Alderete, J.F., 1989. *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. Infect. Immun. 57, 2991–2997.

Bondurant, R.H., Honigberg, B.M., 1994. Trichomonads of veterinary importance. In: Kreier, J.P. (Ed.), Parasitic Protozoa. Academic Press, New York, pp. 111–188.

Fiori, P.L., Rappelli, P., Addis, M.F., Sechi, A., Cappuccinelli, P., 1996. *Trichomonas vaginalis* haemolysis: pH regulates a contact-independent mechanism based on pore-forming proteins. Microb. Pathog. 20, 109–118.

Friedhoff, T., Kuhnigk, C., Müller, I., 1991. Experimental infections in chickens with *Chilomastix gallinarum*, *Tetratrichomonas gallinarum*, and *Tritrichomonas eberthi*. Parasitol. Res. 77, 329–334.

Garber, G.E., Lemchuk-Favel, L.T., Bowie, W.R., 1989. Isolation of a cell-detaching factor of *Trichomonas vaginalis*. J. Clin. Microbiol. 27, 1548–1553.

Gentry, A., Lawrence, N., Lushbaugh, W., 1985. Isolation and differentiation of herpes simplex virus and *Trichomonas vaginalis* in cell culture. Clin. Microbiol. 22, 199–204.

Gerhold, R.W., Yabsley, M.J., Smith, A.J., Ostergaard, E., Mannan, W., Cann, J.D., Fischer, J.R., 2008. Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. J. Parasitol. 94, 1335–1341.

Gerhold, R.W., Allison, A.B., Sellers, H., Linnemann, E., Chang, T.H., Alderete, J.F., 2009. Examination for double-stranded RNA viruses in Trichomonas gallinae and identification of a novel sequence of a Trichomonas vaginalis virus. Parasitol. Res. 105, 775–779.

Gilbert, R.O., Elia, g., Beach, D.H., Klaessig, S., singh, B.N., 2000. Cytopathogenic effect of *Trichomonas vaginalis* on human vaginal epithelial cells cultured *in vitro*. Infect. Immun. 68, 4200–4206.

Goldman, M., Honigberg, B.M., 1968. Immunologic analysis by gel diffusion technics of the effects of prolonged cultivation on *Trichomonas gallinae*. J. Protozool. 15, 350–352.

Grabensteiner, E., Bilic, I., Kolbe, T., Hess, M., 2010. Molecular analysis of clonal trichomonad isolates indicate the existence of heterogenic species present in different birds and within the same host. Vet. Parasitol. 172, 53–64.

Honigberg, B.M., Becker, D., Livingston, C., Mclure, T., 1964. The behavior and pathogenicity of two strains of *Trichomonas gallinae* in cell culture. J. Protozool. 11, 447–465.

Karen, C., Meysick, K., Garber, G., 1990. Growth of *Trichomonas vaginalis* in a serum-free McCoy cell culture system. J. Parasitol. 76, 926–928.

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037

Kawaguchi, T., Nomura, K., Hirayama, Y., Kitagawa, T., 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. 47, 4460–4464.
Krieger, J.N., Ravdin, J.I., Rein, M.F., 1985. Contact-dependent cytopathogenic mechanisms of *Trichomonas vaginalis*. Infect. Immun. 50, 778–786.
Kulda, J., Honigberg, M., 1969. Behavior and pathogenicity of *Tritrichomonas foetus* in chick liver cell cultures. J. Protozool. 16, 479–495.
Kulda, J., 1967. Effect of different species of trichomonads on monkey kidney cell cultures. Folia Parasitol. 14, 295–310.
Mehlhorn, H., Al-Qurashi, S., Amin, A., Hess, M., 2009. Fine structure of the bird parasites Trichomonas gallinae and Tetratrichomonas gallinarum from culture. Parasitol. Res. 105, 751–756.
Moscovici, C., Moscovici, M.G., Jimenez, H., Lai, M.M., Hayman, M.J., Vogt, P.K., 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell 11, 95–103.
Narcisi, M., Sevoian, M., Honigberg, B.M., 1991. Pathologic changes in pigeons infected with a virulent *Trichomonas gallinae* strain (Eiberg). Avian Dis. 35, 55–61.
Neimanis, A.S., Handeland, K., Isomursu, M., Agren, E., Mattsson, R., Hamnes, I.S., Bergsjø, B., Hirvela-Koski, V., 2010. First report of epizootic trichomoniasis in wild finches (family Fringillidae) in southern Fennoscandia. Avian Dis. 54, 36–41.
Norton, A., 1997. Pathogenicity of a strain of *Trichomonas gallinarum* in turkeys and its possible interaction with cecal coccidia. Avian Dis. 41, 670–675.
Peterson, K.M., Alderete, J.F., 1984. *Trichomonas vaginalis* is dependent on uptake and degradation of human low density lipoproteins. J. Exp. Med. 160, 1261–1272.
Pindak, F.F., Pindak, M.M., Gardner, W.A., 1993. Contact-independent cytotoxicity of *Trichomonas vaginalis*. Genitourin Med. 69, 35–40.
Robinson, R.A., Lawson, B., Toms, M.P., Peck, K.M., Kirkwood, J.k., Chantrey, J., Clatworthy, I.R., Evans, A.D., Hughes, L.A., Hutchinson, O.C., John, S.K., Pennycott, W., Perkins, M.W., Rowley, P.S., Simpson, V.R., Tyler, K.M., Cunningham, A.A., 2010. Emerging infectious disease leads to rapid population declines of common British birds. PLoS One 5, e12215.
Silva-Filho, F.C., de Souza, W., 1988. The interaction of *Trichomonas vaginalis* and *Tritrichomonas foetus* with epithelial cells *in vitro*. Cell Struct. Funct. 13, 301–310.
Sommer, U., Costello, C.E., Hayes, G.R., Beach, D.H., Gilbert, R.O., Lucas, J.J., Singh, B.N., 2005. Identification of *Trichomonas vaginalis* cysteine proteases that induce apoptosis in human vaginal epithelial cells. J. Biol. Chem. 280, 23853–23860.
Stabler, M., 1954. *Trichomonas gallinae*: a review. Exp. Parasitol. 3, 368–402.
Tyzzer, E., 1920. The flagellate character and reclassification of the parasite producing "blackhead" in turkeys-*Histomonas* (gen.nov.) *meleagridis* (Smith). J. Parasitol. 6, 124–131.
Work, T., Hale, J., 1996. Causes of owl mortality in Hawaii, 1992 to 1994. J. wildl. Dis. 32, 266–273.

Please cite this article in press as: Amin, A., et al., *Trichomonas gallinae*, in comparison to *Tetratrichomonas gallinarum*, induces distinctive cytopathogenic effects in tissue cultures. Vet. Parasitol. (2011), doi:10.1016/j.vetpar.2011.11.037