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Influence of culture medium pH on internalization, growth and phenotypic plasticity of *Neospora caninum*

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ABSTRACT

Neospora caninum, a strictly intracellular protozoan, is a major leading cause of parasite-induced abortion in cattle. A widely held view of *N. caninum* infection is that both cellular proliferation and stage interconversion (tachyzoite–bradyzoite transformation) are triggered, perhaps even modulated by, changes in cultural conditions. This study tested the hypothesis that exposure of *N. caninum* tachyzoites to different pH culture media affects the parasite's entry, proliferation and cyst formation in cultured cells. The endocytic pathway for *N. caninum* entry into the K562 cell line was found to be mediated by low pH of culture medium. Internalization of *N. caninum* by host cells was significantly increased in acidic and alkaline culture medium compared to cells maintained in neutral medium as revealed by transmission electron microscopy. Parasite proliferation within Vero cells was assessed by plaque formation assay and was found to be highest when pH level was optimum, paralleled by a decrease in the number of cysts. In contrast, parasite encystation increased when the pH level was alkaline or acidic, as evaluated by indirect immunofluorescence and immunocytochemical analyses. Acidic pH regardless of state of host cell infection suppressed the rate of host cell division. These findings suggest that culture medium pH has a determinable effect on the host cell-*N. caninum* interaction and support the hypothesis that pH of culture medium influence the entry, growth, and phenotypic plasticity of *N. caninum* in mammalian cells.

1. Introduction

Neosporosis is an economically important disease of cattle and carnivores caused by the apicomplexan protozoan parasite *Neospora caninum* (Dubey et al., 2007). Effective prevention and treatment of neosporosis requires full understanding of the parameters that determine the entry, growth and survival of *N. caninum* in host cells. Infection by all apicomplexan parasites requires entry of the organism into the vertebrate host cell by attaching to and then creating an invagination in the host cell plasma membrane, and involves different protozoal ligands and cellular membrane receptors (Hemphill, 1996; Carruthers et al., 2000; Naguleswaran et al., 2002; Hemphill et al., 2006). Internalization and intracellular survival are critical mechanisms by which these organisms subvert the host's defence mechanism via providing the parasite with a protective niche against the host's immune response and against parasiticides that do not penetrate into host

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cells. Much of the knowledge regarding internalization and intracellular survival of apicomplexan protozoa has been gained from studies on *Toxoplasma gondii* (Botero-Kleiven et al., 2001; Robibaro et al., 2001). In contrast, very little is known about these critical events in *N. caninum* infection.

An intriguing feature of *N. caninum* and other cyst-forming apicomplexan protozoa is their autoreactivity: following exposure to stress, tachyzoite transforms from a growing state to a resting bradyzoite-containing cyst (Soete et al., 1994; Risco-Castillo et al., 2004; Hemphill et al., 2006; Ferreira da Silva Mda et al., 2008; Eastick and Elsheikha, 2010). This developmental differentiation is hypothesized to occur as an adaptive process allowing the parasite to cope with the adverse environments, and can be mediated or influenced by the complex cellular environment. Also, stage transformation process involves several paradoxical mechanisms that are meant for protection of the host but exploited by the parasite for its survival (Lyons et al., 2002; Elsheikha and Morsy, 2009).

Despite the large body of evidence available about the pathogenesis of neosporosis, the cellular mechanisms for *N. caninum* entry, growth and cyst formation are largely unexplored. Among the factors influencing the dimorphic switching of apicomplexan protozoa, pH of the culture medium is rarely studied in *N. caninum* (Soete et al., 1994; Weiss et al., 1995, 1999). In the present study, we investigate the hypothesis that the culture medium pH can govern the internalization, growth and cyst formation of *N. caninum*, knowledge which is needed to address the pathogenesis of a disease with significant economic implications.

2. Materials and methods

2.1. Cell lines and culture conditions

K562 cells, a human lymphoma cells were purchased from the Health Protection Agency Culture Collection (HPACC, Salisbury, Wiltshire, UK) and cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotic–antimycotic mixture. African Green Monkey kidney cell line (Vero) was purchased from the HPACC and used between passages 17 and 19. Cells were grown continuously as a monolayer in 75-cm^2 cell culture flasks in 15 ml of complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5–10% heat inactivated FCS (Invitrogen, GIBCO, UK), 25 mM HEPES, 2 mM L-glutamine, 10,000 U/ml penicillin G sodium, 10,000 μg/ml dihydrostreptomycin, 250 μg/ml amphotericin B, nonessential amino acids, and 100 mM sodium pyruvate. Cells were grown in the growth medium (10% FBS) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air until the monolayer became confluent; then the medium was changed to maintenance medium (5% FCS). Stock cultures were maintained by lifting the monolayers with trypsin–EDTA (0.25% trypsin with 1 mM EDTA·4Na) once a week and transferring the cells to new culture flasks. To exclude if cell viability could be regarded as a factor effecting parasite invasion and therefore any subsequent induced interconversion, viability of cells was assessed using trypan blue exclusion assay prior to inoculation onto culture plates or flasks.

2.2. The parasite

N. caninum (Nc-Liverpool) strain was a gift from Professor Sandy Trees (University of Liverpool) and was used between passages 30 and 33. Parasite stock cultures were maintained in Vero cells for long periods by varying the FCS concentration. Parasites were harvested from their feeder cell culture and purified as described previously (Elsheikha et al., 2006). The number of tachyzoites was estimated using a haemocytometer. The final volume of suspension was adjusted so that 1×10^4 and 1×10^5 tachyzoites ml^{-1} of culture medium were used for the inoculation of Vero cells in 6-well plate and in culture flask assays, respectively. All experiments were conducted in triplicate.

2.3. Endocytosis assay

The effect of extracellular medium pH on the rate of endocytosis was determined on K562 cells infected with *N. caninum*, using FM2-10 dye. This dye has a fluorescence intensity that increases upon membrane incorporation. This property has been used to measure endocytosis, i.e. kinetic of plasma membrane internalization (Rauch and Farge, 2000). Therefore the kinetic of endocytosis can be obtained by measuring the increases in fluorescence intensity as a function of time. For this, approximately, $25 \times l0^6$ K562 cells/ml RPMI medium were infected with the parasite at a ratio of (1:1) and incubated at 37 °C for 2 h in media with acidic (6.5), neutral (7.5) and alkaline (8.5) pH. Different DMEM at pH values of 6.5 and 8.5 were made by using hydrochloric acid and sodium hydroxide, respectively. The cells were pelleted by centrifuging at 1000 rpm for 5 min, and the supernatant was removed. The cells were then washed once with sterile phosphate buffered saline (PBS). The cells were then centrifuged at 13,000 rpm for another 30 s. The supernatant was removed and the cell pellet resuspended in 1 ml of FM2-10 staining solution (FM2-10 final concentration 1 μM). After this 100 μl of the stained cells were transferred on a 96 well plate, and fluorescence (excitation 530 nm, emission 590 nm) was measured using the FLUROstar Optima BMG (Labtech, Germany). From the fluorescence intensity measured as a function of time, the kinetic of membrane internalization was deduced. In general, K562 cells display a kinetic of endocytosis in resting conditions of about 0.05%/s (Rauch and Farge, 2000).

2.4. Evaluation of N. caninum internalization

The rate of *N. caninum* internalization within K562 cells incubated in different pH culture media was assessed using transmission electron microscopy (TEM). K562 cells were infected with *N. caninum* and incubated in neutral, acidic, or alkaline medium for 2 days. Cells of each treatment were centrifuged and supernatants were discarded. The cell pellets were fixed overnight with 2.5% glutaraldehyde at 4 °C, post-fixed with 1% (w/v) OsO_4 for 1 h, dehydrated in increasing concentration of ethanol and embedded in Spurr's resin. Ultrathin sections were stained with 0.2% uranyl acetate and contrasted with lead citrate. The samples were observed using a JEM-1230 electron microscope (JOEL) and MegaView Software.

2.5. Effects of pH on kinetics of parasite growth and encystation

In one experiment, Vero cells were cultured in 6-well plates with cover slips, maintained with DMEM at a neutral pH until 60–70% confluency. Media were prepared as previously mentioned so that pH values of 6.5, 7.5 and 8.5 were obtained. Neutral medium with a pH of 7.5 was used as a control. Three equal numbers of free tachyzoites (1×10^4/ml) harvested from previously infected cultures were suspended in one of the 3 different media and incubated for 1 h exposure period, after which all parasite groups were washed and re-suspended in neutral media. The parasites were transferred to their corresponding labelled wells of plated cells, depending on the treatment they received, at a 1:3 host–parasite ratio. Additional neutral maintenance media was added. All medium treatment conditions were tested in triplicate.

In a second experiment, Vero cell cultures were established in three 75 cm^2 tissue culture flasks, which were infected with 1×10^5 tachyzoites ml^{-1} at a 1:3 host–parasite ratio. Medium at each pH value (i.e. 6.5, 7.5, or 8.5) was exchanged with the maintenance media of each infected flask. The flasks were incubated for 4 h, after which the media was changed so that all flasks contained neutral complete DMEM. The flasks were re-incubated until the end of the experiment.

From here onwards, the day of pH treatment will be referred to as day 0, with days 1–7 referring to days post pH treatment. The infected cells were monitored daily and any changes or differences between cultures were recorded. At the first appearance of parasitic lesions, the number of lesions present within 20 microscopic fields (20×) was recorded daily for each treated culture. The time at which the experiments were ended was dependent on parasite aggression. When ended, the number of free zoites per volume of media for each pH treatment was calculated using a haemocytometer.

2.6. Immunodetection of N. caninum stage transformation

Transition of free tachyzoites to tissue cysts was monitored by indirect immuno-fluorescence (IFA) and immunocytochemical assays, for tachyzoites and tissue cysts, respectively. For the detection of tachyzoites, monolayers of Vero cells on poly-L-lysine coated coverslips in 12-well cell culture plates (Costar) were infected with *N. caninum* tachyzoites at a 1:1 host–parasite ratio. Tachyzoites were allowed to bind and invade the host cells for 2 h, and then the remaining extracellular tachyzoites were removed by replacing the media with fresh media. The culture plates were allowed to continue until the end of the experiment. At 1, 2, and 3 day post infection, two wells were rinsed three times with tris buffered saline (TBS). The cells were then fixed with cold acetone:methanol (1:1) for 10 min. Fixed cells were incubated with primary monoclonal mouse anti-NcSAG1 antibody (a kind gift from Dr. Andrew Hemphill, University of Zurich, Zurich, Switzerland) in 1:400 dilution for 2 h at room temperature (RT). Cells were rinsed three times in TBS before incubating with a secondary goat-anti-mouse IgG antibody conjugated with Alexa Fluor 488 (FITC filters) (Invitrogen Ltd, Paisley, UK) at 1:500 dilution in TBS for 2 h in dark at RT. After removing excess fluid, cells were rinsed three times in TBS. The coverslips were carefully removed from the cell culture plates and were mounted with ProLong Gold antifade reagent with 4′,6-diamino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) on microscopic slides. Negative controls were performed without the primary reagents.

Cysts of *N. caninum* in infected cells were detected by immunocytochemical staining with a primary monoclonal mouse anti-CC2 IgG antibodies (a gift from Dr. Andrew Hemphill, Switzerland) followed by visualization with envision + system-HRP (DAB) for mouse IgG (DAKO Corporation, Carpinteria, CA) following the manufacturer's instructions. Briefly, after decanting the medium the Vero cells were rinsed gently with TBS and endogenous peroxidase was blocked by incubating the cells with peroxidase for 10 min. Then, cells were incubated with 1:200 dilution of the primary antibody for 40 min at RT. Controls were performed with TBS with no primary antibody. After removing the excess fluid the cells were rinsed with TBS and incubated with appropriate labelled polymer (anti-mouse IgG + HRP) for 40 min at RT. After removing excess fluid cells were rinsed gently with TBS and incubated with substrate-chromogen solution for 10 min at RT. After rinsing the cells with TBS, cells were counterstained with haematoxylin for 1 min and rinsed with water. Both stages of parasite development (i.e. tachyzoite and cyst) were visualized by using a Leica DMIL microscope connected to Leica application suite for image analysis version 2.8.1 (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

2.7. Host cell cycle analysis

Cell cycle analysis was performed as described previously (Riccardi and Nicoletti, 2006) in order to determine potential side effects of the pH of culture medium and infection with *N. caninum* on K562 cell proliferation at 1 day and 2 day posttreatment. Briefly, K562 cells were centrifuged at 1200 rpm for 10 min and the supernatants were discarded. Cells were washed with 10 ml of cold (4 °C) PBS containing 1 mg/ml glucose and centrifuged at 1200 rpm for 10 min. After careful removal of the majority of remaining supernatant, the pellets were resuspended by vigorous vortexing for 10 s. Cells were fixed in 70% ethanol for a minimum of 18 h at 4 °C. Ethanol fixed cells were then stained with propidium iodide (PI) staining solution containing 50 μg of PI, 100 kunitz units of ribonuclease A (Sigma) and 1 mg glucose in 1 ml of PBS (freshly prepared for each assay). The cell mixture was then transferred to flow cytometer tubes (BD Falcon), capped and incubated at RT on a rocking platform for 30–90 min in the dark. Stained cells were analyzed within 24 h using BD FACS CantoII (BD Biosciences) flow cytometer and the data was analyzed using BD FACS DIVA software. Twenty thousand cells were analyzed for each sample using the 488 nm laser for excitation and red fluorescence (>600 nm) and side scatter were measured.

Voltages were adjusted using PI-stained untreated cells and the same settings were used for all the samples.

2.8. Statistical analysis

A one-way analysis of variance was calculated to determine if there were any significant differences between the mean numbers of parasite lesions calculated for each treatment. This statistical test calculates the significance of differences while assuming a normal distribution of results. Normality tests were performed for each set of results to determine whether the results were in fact normally distributed. Treatment groups were considered statistically different from controls at $P < 0.05$. In order to quantify the association between the change in the percentage of mitotic cells between day 1 and day 2 according to pH and infection, a linear regression model was constructed using the l m function in (R Development Core Team, 2009).

3. Results

3.1. Acidic pH increases parasite endocytosis

As shown in Fig. 1, when cells are infected with the parasite in alkaline culture environment, the rate of membrane internalization expressed in %/s increases significantly ($P < 0.05$), but, membrane internalization was even much prominent in infected cells incubated in acidic culture environment ($P < 0.01$). Interestingly, this increase is not related solely to the pH as low pH or high pH alone has no significant effect on membrane internalization. Therefore, it is the interaction between the parasite and the cell that drives such a kinetic in endocytosis.

3.2. Influence of medium pH on parasite internalization

Effect of different culture medium pH on the parasites' entry was addressed by electron microscopy. As shown in Fig. 2, a two-fold increase in parasite number was observed when comparing K562 cells growing in an acidic or alkaline condition against neutral pH condition. On the other hand, a number of tachyzoites were noted outside the K562 cells incubated in neutral medium, whereas no single parasite was noted outside the cells in case of treatment with alkaline or acidic culture medium. No remarkable ultrastructural changes were noted in *N. caninum* or K562 cells subjected to different pH treatments.

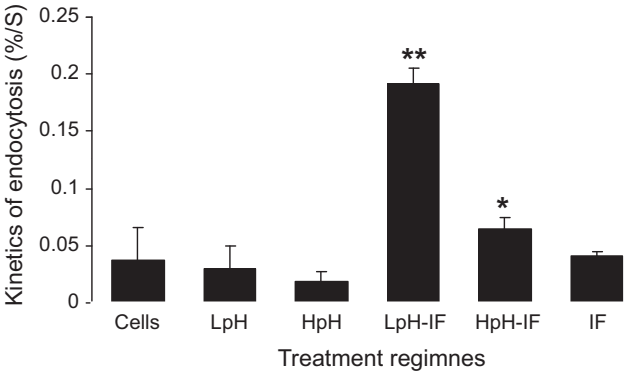

Fig. 1. Results of endocytosis measurements of K562 cells infected or non-infected with *Neospora caninum* and incubated in acidic, neutral, or alkaline medium at 37 °C for 2 h. Abbreviations: cells, K562 cells in neutral medium; LpH, cells treated with low (acidic) pH; HpH, cells treated with high (alkaline) pH; LpH-IF, cells treated with low pH and infected; HpH-IF, cells treated with high pH and infected; IF, infected cells treated with neutral medium. Values are averages ± standard deviations of results from three independent experiments. Two-tailed Student's *t* tests were used for statistical analysis; * indicates a *P* value of <0.05, and ** indicates a *P* value of <0.01.

3.3. Plaque formation efficiency

All cell cultures were calculated to be at least 96% viable. Free zoites were visible in all infected cultures until day 3 post-treatment. At this time, lesions became visible as areas of heavy infection, where there were colonies of zoites present. These lesions were often in the form of parasitic plaques (zoites surrounded areas of cell destruction). There was an increase in the mean number of lesions observed over time for each treatment (Fig. 3). Only four sets of results were deemed not to follow a normal distribution (Table 1), though the analysis of variance calculated may not have been as accurate as a result of this. Assuming that the effect of this is minimal, the analysis of variance demonstrated that there is a significant difference between the means of each treatment group on any one day and therefore that these results were unlikely to be due to chance. It is probable that the different treatment conditions influenced the mean number of lesions recorded daily for each group.

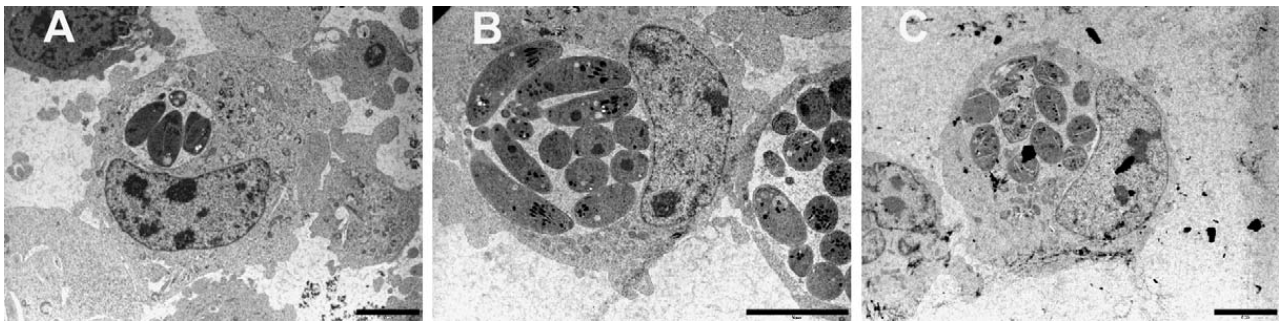

Fig. 2. TEM micrographs of K562 cells infected with *Neospora caninum* and exposed to (A) neutral, (B) acidic, or (C) alkaline culture medium. Low and high pH treatments showed at least a two-fold increase of internalization of *N. caninum* tachyzoites compared to cells treated with neutral medium. Scale bar: (A) 2 μm; (B) 5 μm; (C) 2 μm.

Table 1
The mean lesion number recorded and standard deviation of results for each day post treatment. *P*-values as calculated by one-way analysis of variance are also given for each day.

	Treatment (mean ± SD)			*P*-value	Normal distribution
	Acidic	Neutral	Alkaline		
Extracellular treatment					
Day 3	1.45 ± 1.00	4.10 ± 1.21	1.45 ± 1.15	<0.00	Yes
Day 4	2.65 ± 1.04	4.85 ± 1.14	1.75 ± 1.21	<0.00	Yes
Day 5	4.25 ± 1.45	7.1 ± 1.41	3.15 ± 1.09	<0.00	Yes
Day 6	6.20 ± 0.95	9.45 ± 1.00	5.00 ± 1.21	<0.00	Only alkaline abnormal
Day 7	7.90 ± 0.97	11.45 ± 1.00	7.05 ± 1.00	<0.00	Yes
Intracellular treatment					
Day 3	1.90 ± 1.52	3.05 ± 1.40	1.85 ± 1.14	0.01	Yes
Day 4	2.80 ± 0.83	4.35 ± 1.42	2.85 ± 1.04	<0.00	Only neutral abnormal
Day 5	7.35 ± 1.81	8.40 ± 1.63	7.50 ± 1.32	<0.09	Only neutral abnormal
Day 6	9.70 ± 1.26	13.60 ± 1.14	10.15 ± 1.39	<0.00	All abnormal

3.4. Tachyzoite production and cyst formation

As more cell destruction occurred within the cultures, more free tachyzoites became apparent. The cell confluency decreased from day 3 when lesions were first observed. The free zoites observed from this time were the same in appearance to those observed prior to the appearance of lesions. When the cell confluency had decreased to less than 50% for all extracellular/intracellular treated cultures, the experiment was ended. This was the case at day 6 for the intracellular treated cultures. The total number of free tachyzoites present within media harvested from each of the treatment flasks was calculated using a haemocytometer. Control cultures with neutral medium recorded the highest number of zoites 11.4×10^6, followed by 8.7×10^6 in acidic treated culture and 5.4×10^6 in the alkaline treated culture. The cell confluency of all extracellular treated cultures reached less than 50% on day 7 post treatment. The media was harvested from wells of each treatment and the free tachyzoites per volume of harvested media was calculated. Again, most free zoites were present in the neutral media 0.54×10^6 followed by 0.175×10^6 in the acidic treated culture, and 0.06×10^6 in the alkaline treated culture. The presence of morphologically similar tissue cysts in Vero cells of both alkaline and acidic treated cultures was confirmed using IFA and immunocytochemistry (Fig. 4). Cyst size ranged from 10 to 20 μm in diameter. Tissue cysts were not observed in neutral treated cultures.

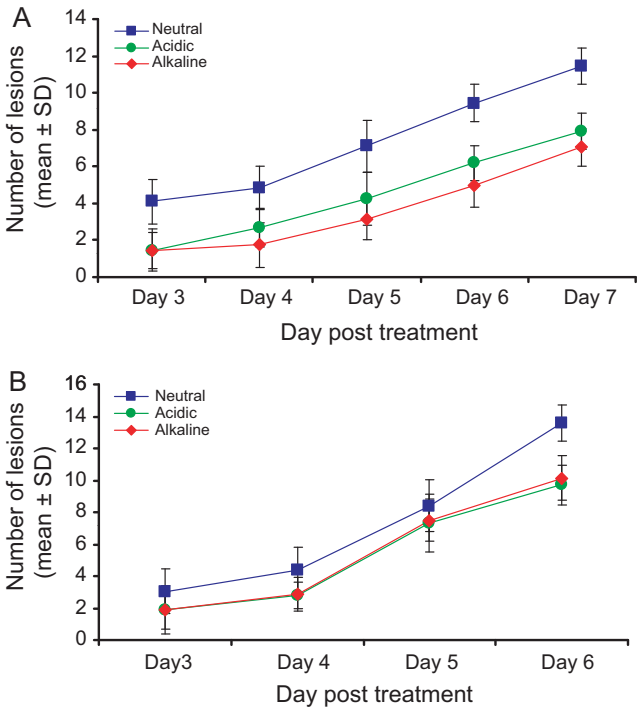

Fig. 3. Effects of the culture media pH on the kinetics of induction of plaque formation in Vero cell monolayer by *Neospora caninum* in response to different pH of culture medium. Tachyzoites were exposed to pH while they are extracellular (A) or intracellular (B).

3.5. Cell cycle alterations

We studied the growth cycle of K562 cells in regard to infection and/or exposure to different pH culture media. Changes in the percentage of dividing (mitotic) cells between day 1 and day 2 are presented in Fig. 5. The percentage of mitotic cells was lower on day 2 except for 'Alkaline/Not infected' treatment for which it increased slightly. There was a significant effect of day ($p < 0.01$), pH ($p < 0.05$) but not of infection ($p = 0.17$) on the percentage of mitotic cells (Table 2). Interestingly, the decrease in cell mitosis occurred in acidic culture medium 2 day post treatment whether or not cells were infected with *N. caninum*.

4. Discussion

In this study we described changes in the rate of *N. caninum* internalization by host cells in response to the modification of culture medium pH. The apparent enhance-

Table 2
Results of the linear model for the association between the percentage of mitotic cells and day, pH and infection status. The intercept of the model refers to the uninfected/neutral pH on day 1 modality.

	Estimate	Std. error	*t* value	Pr(>|*t*|)
Intercept	10.2	0.688385	14.81729	1.30E−15
Day	−4.20556	0.61571	−6.83041	1.18E−07
pH acidic	1.841667	0.754088	2.442244	0.020496
pH alkaline	1.8	0.754088	2.38699	0.023272
Infected	0.872222	0.61571	1.416612	0.166568

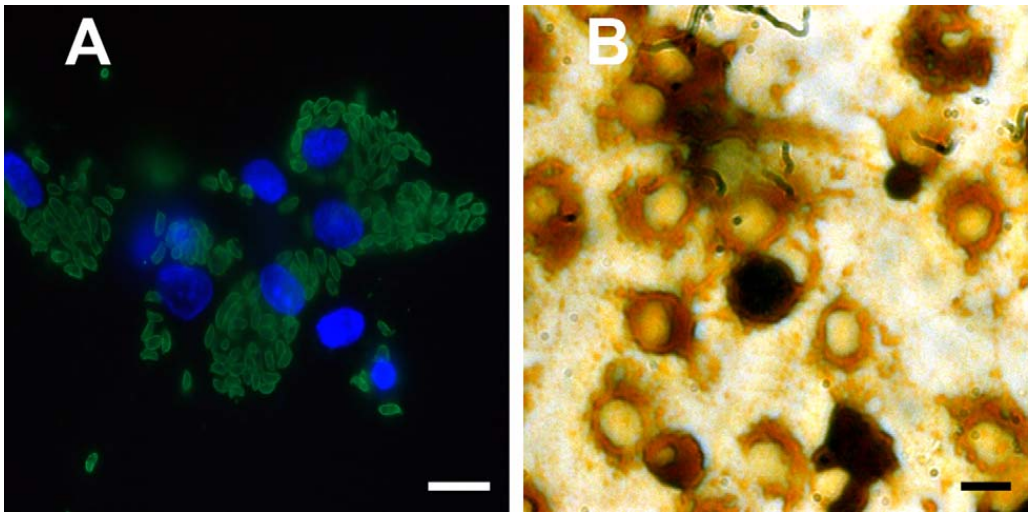

Fig. 4. The cyst formation of *Neospora caninum* in Vero cell culture is pH-dependent. (A) Only free tachyzoites were detected in cells treated with neutral medium. (B) *N. caninum* cyst observed after exposure to acidic pH. Scale bar = 20 μm.

ment in *N. caninum* endocytosis (Fig. 1) and internalization (Fig. 2) is intriguing, but the exact mechanism of action of the pH-induced enhancement of endocytosis is largely undefined. It is likely that acidic environment stimulate the generation of cellular environment more permissible for *N. caninum* infection. Alternatively, it might be envisaged that extracellular acidic environment trigger conformational changes of the parasite coat or host cell surface receptors, or alter the functional properties of cell surface molecules/de novo protein, modulating specific steps involved in host–pathogen interactions and membrane internalization. Consistent with this interpretation, infected cells treated with neutral pH or non-infected cells treated with low or high pH did not show any effect on the rate of endocytosis. Taken together, these data indicate that pH-induced changes leading to parasite entry are dependent of the presence of the parasite and host cells in acidic environment. However, we cannot exclude the possibility that other factors besides a low pH are involved in or required for the low-pH enhanced endocytosis.

We have shown that culture medium pH can alter the rate of proliferation and differentiation of *N. caninum*, such that *N. caninum* grown in the neutral culture medium have a higher rate of proliferation relative to *N. caninum* cultured in acidic or alkaline medium. Additionally, *N. caninum* also demonstrated a pH effect on parasite transformation, which is consistent with that observed for *T. gondii* (Ferreira da Silva Mda et al., 2008). These observations suggest that *N. caninum* possesses mechanism(s) of adaptation that enable optimal activity of each life stage at the corresponding environmental pH. The mean number of lesions recorded was always higher for the neutral treated cultures at any one time (Fig. 3). This finding was expected as this was more optimal condition for parasite growth, and the *N. caninum* tachyzoites were able to successfully thrive in specific areas of the Vero cells. The acidic treated culture generally showed higher mean lesion numbers than the alkaline treated culture. These findings illustrate that acidic conditions allowed greater parasitic proliferation than alkaline conditions. This was exacerbated when the cells were treated extracellularly, probably due to the fact that modified media was in direct contact with the tachyzoites. There was little difference in the rate of increase in lesion numbers between treatments, though between days 5 and 6 post treatment the intracellular experiment shows a slightly higher rate of increase in the neutrally treated culture than in the acidic and alkaline treated cultures (Fig. 3). This may be due to the long term effect that the detrimental pH conditions had on the cells or parasite itself. We can also assume that stage conversion is highest in alkaline conditions, as this study observed less free forms of the parasite under these conditions.

The IFA and immunocytochemical tests demonstrated that encystation had occurred in both the acidic and alkaline extracellular treated cultures (Fig. 4). No cysts were found in neutral treated cultures. Average cyst size was consistent with previous finding (Jardine, 1996). The number of free zoites recorded was highest in the neu-

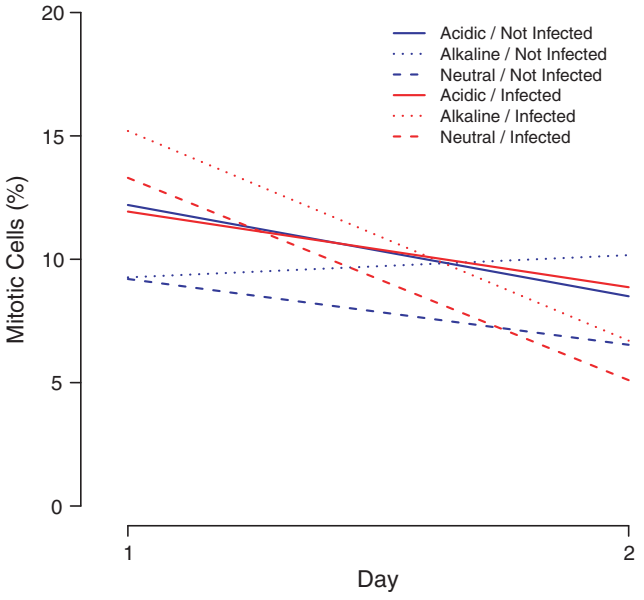

Fig. 5. Variation in the percentage of K562 mitotic cells between day 1 and day 2 for the 6 treatment groups. There were 3 repetitions in each group.

tral extracellular and intracellular treated cultures. It was expected that neutral treated cultures would contain more free tachyzoites than alkaline cultures due to a proportion of the free tachyzoites present in alkaline cultures invading cells and forming bradyzoite-containing tissue cysts, such as those found in previous studies (Soete et al., 1994; Risco-Castillo et al., 2004), once alkaline treatment was applied. However, whether the stress caused by acidic pH was enough to cause significant encystation was unknown, though it is likely that some significant encystation had occurred in this study as free zoites were not as numerous as for neutral treated cultures, a finding which was also supported by ultrastructural analysis using TEM. It appears that the results of the free zoite counts support the results of the mean lesion counts as the acidic pH treatment did not demonstrate parasite inhibition as evidently as alkaline pH treatment. If the assumption that a small number of free tachyzoites is reflective of a larger number of bradyzoite containing tissue cysts is correct, these results support the earlier findings (Risco-Castillo et al., 2004), where a relatively large number of bradyzoite-containing cysts were present in alkaline treated cells compared to neutral treated cells.

Knowledge of the conditions which induce the development of cystic stage from tachyzoite stage and therefore contribute to *N. caninum* persistence is increasing with the use of *in vitro* model of stress-induced stage conversion (Eastick and Elsheikha, 2010). Evidences obtained from *in vitro* experiments indicate that stress triggers a developmental transformation of *N. caninum* and the related parasite, *T. gondii* (Soete et al., 1994; Ferreira da Silva Mda et al., 2008). As expected, our findings indicate that cyst formation in *N. caninum* can be pH-dependent. However, the present study is different to most which have applied pH to assess stage transformation because parasite infected cultures are generally maintained in an alkaline medium over the experimental period. Herein, *N. caninum* tachyzoites were briefly exposed to acidic or alkaline treatments to assess stage conversion in comparison to neutral treatment as a control. After treatment, all cultures were maintained in neutral media. This meant that the experiment could be carried out for a longer period of time because the cells were not damaged by maintenance in detrimental culture conditions. Due to the virulence of the NC-Liverpool strain used, the lesion formation could only be observed for up to seven days post treatment because cell confluency decreased to 50% and the experiment was ended. Perhaps the use of a less virulent strain would have provided results over a longer time period.

Further, we examined the relationship between extracellular pH and parasite infection, and its impacts on the growth cycle of K562 cells. Infection with *N. caninum* regardless of the type of pH of the culture media decreased the mitosis of infected cells (Fig. 5). *N. caninum* is adapted to grow and develop only within the host cell cytoplasm. Therefore, this intracellular parasite must use tactics to ensure a replication-permissive niche. From an evolutionary point of view *N. caninum* seems to suppress the host cell cycle because any cell division of infected cells will threaten the parasites' survivability. The inhibitory effect of protozoan infection on host cell mitosis has been documented in other protozoa e.g. *Encephalitozoon microsporidia* (Scanlon et al., 2000). Interestingly, our findings showed that acidic pH regardless of state of host cell infection suppress the rate of host cell division.

Together, the above observations indicate that proliferation of *N. caninum* is significantly higher when pH level is optimum, and that parasite encystation occurs when the pH level is not favourable. How then does pH exert its effects on the frequency of stage transformation? It is possible that the differences in tachyzoite production are conditioned by their capacity of infection in the host cells and not by variations in tachyzoite proliferation after exposure to different pH treatment. Also, it is likely that variations in pH can trigger changes in gene expression that result in changes in the parasite's development. This study provides a starting point in efforts to characterize the molecular basis for pH-induced endocytosis and encystation in *N. caninum*. A better understanding of the mechanisms by which pH modulates the host–parasite interaction at the membrane interface in *N. caninum* infection has important implications for the development of therapeutic approaches to treat neosporosis.

Conflicts of interest

The authors declare no conflicts of interest.

Ethics

No animal experimentation has been undertaken.

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