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Comparison of host cell invasion and proliferation among *Neospora caninum* isolates obtained from oocysts and from clinical cases of naturally infected dogs

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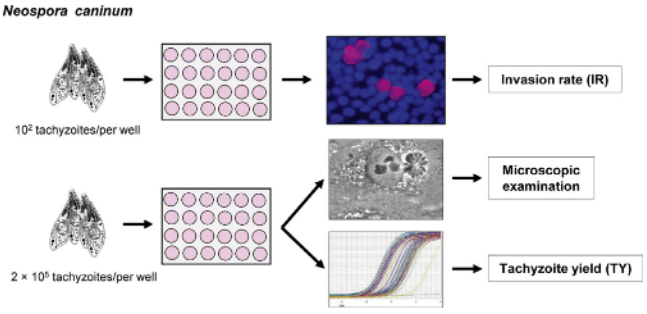
HIGHLIGHTS

- Tachyzoite intracellular proliferation is associated with *N. caninum* pathogenesis.
- Tachyzoite proliferation capacities have been related to bovine isolate virulence.
- Invasion and tachyzoite yield *in vitro* were evaluated in canine isolates.
- Differences were found between isolates from oocysts and from dogs with signs.
- Correlation was found between proliferation capacities and virulence in mice.

GRAPHICAL ABSTRACT



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ABSTRACT

In a previous study we have shown that the *in vitro* invasion rate (IR) and tachyzoite yield (TY) are associated with the virulence phenotypes of *Neospora caninum* isolates of bovine origin. In addition, we recently observed marked differences in virulence when canine isolates were compared in a pregnant BALB/c mouse model. In this study, we investigated whether invasion and proliferation capacities could be used as virulence-related *N. caninum* phenotypic traits. Of the isolates compared in mice, four canine isolates obtained from oocysts (Nc-Ger2, Nc-Ger3, Nc-Ger-6, Nc-6 Arg) had shown a low-moderate virulence, and two further isolates obtained from dogs with neurological signs (Nc-Bahia, Nc-Liv) were highly virulent. The IR for each isolate was determined by a plaque assay and the counting of immunofluorescence-labeled parasitophorous vacuoles at 3 days post-inoculation (p.i.). The TY was determined by the quantification of tachyzoites at 56 h p.i. by real-time PCR. Most of the canine isolates showed similar IR values under controlled invasion conditions for 4 h and 72 h p.i., indicating a limited time period for invasion similar to that observed for bovine isolates. The Nc-Ger3, Nc-Bahia, and Nc-Liv isolates showed a significantly higher IR and TY than the Nc-Ger2 and Nc-Ger6 isolates ($P < 0.0001$). A correlation was found between the IR$_s$ and TY ($\rho > 0.885$, $P < 0.033$), as well as between the TY and both dam morbidity ($\rho = 0.8452$, $P < 0.033$) and pup mortality ($\rho > 0.8117$, $P < 0.058$) in mice. These results demonstrate the importance both the invasive and proliferative capacities have on the virulence of canine *N. caninum* isolates.

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1. Introduction

Neospora caninum is an obligate intracellular protozoan parasite that is closely related to *Toxoplasma gondii* and causes neuromuscular disease in dogs and abortion in cattle worldwide, leading to significant economic losses in beef and dairy cattle industries (Dubey et al., 2006, 2007; Dubey and Schares, 2011; Reichel et al., 2013). Processes involved in the lytic cycle, including parasite invasion and intracellular proliferation, are essential for the maintenance and multiplication of the parasite *in vitro* and for parasite survival and propagation in host tissues in the course of animal infection (Dubey et al., 2007; Hemphill et al., 2006). Previous studies have demonstrated that there are differences among bovine *N. caninum* isolates in their capacity to produce disease and to be transmitted from dams to offspring in a pregnant BALB/c mouse model (Regidor-Cerrillo et al., 2010; Rojo-Montejo et al., 2009b). Additionally, incidence of infection-induced abortion in cattle has been shown to vary according to the isolate (Caspe et al., 2012; Rojo-Montejo et al., 2009a). The invasion rate (IR) and tachyzoite yield (TY) *in vitro* have been reported to differ among these bovine *N. caninum* isolates and have been associated with mouse pathogenicity, being recognized as virulence-related phenotypic traits (Regidor-Cerrillo et al., 2011). Recently, new populations of isolates were obtained from oocysts and the infected tissues of dogs affected with neurological signs. Examination of these isolates using the same well-established pregnant mouse model revealed marked differences in virulence and vertical transmission (Dellarupe et al., 2014). A clear dichotomy in the capacity to produce disease was found between these isolates: those obtained from oocysts were characterized as having low-moderate virulence, whereas those obtained from the infected tissues of dogs with neurological disease were highly virulent. The aim of the present work was to examine the capacity of these canine isolates to invade host cells and proliferate, as well as to evaluate the potential association between these *in vitro* phenotypic traits and their virulence as previously determined in a pregnant BALB/c mouse model (Dellarupe et al., 2014).

2. Materials and methods

2.1. Description and cultures of N. caninum isolates

This study included four *N. caninum* isolates obtained from oocysts shed by naturally infected dogs, the Nc-Ger2, Nc-Ger3, Nc-Ger-6 and Nc-6 Arg, and two isolates obtained from tissues of naturally infected dogs with neurological signs of neosporosis, the Nc-Bahia and Nc-Liv isolates. A summary of the name, host, geographical origin, passage number in cell culture and pathogenic characteristics in pregnant mice of the isolates included in this study is shown in Table 1. Most of *N. caninum* isolates that were used in these *in vitro* assays were previously subjected to re-isolation from mice to maintain similar passage numbers across evaluated isolates and to minimize the effect of culture adaptation (Bartley et al., 2006) (Table 1).

N. caninum isolates were routinely maintained in a monolayer culture of MARC-145 cells after reactivation from cryovials as described previously (Regidor-Cerrillo et al., 2010). Tachyzoites used in the *in vitro* assays were harvested from MARC-145 cultures when the majority of the parasites were still intracellular (at least 80% of the parasite vacuoles were undisrupted in the cell monolayer) and purified from host cells as described previously (Regidor-Cerrillo et al., 2011). The number of purified tachyzoites was then determined by trypan blue exclusion followed by counting in a Neubauer chamber. Tachyzoites were resuspended at the required doses in Dulbecco Minimum Essential Medium (DMEM) supplemented with a 2% antibiotic–antimycotic solution (Gibco BRL, Paisley, UK) and 2% heat-inactivated fetal bovine serum (FBS) for inoculation on MARC-145 cell cultures. The FBS employed in all *in vitro* assays was from the same production batch *N. caninum*-seronegative by immunofluorescence assay test. All inoculations in assays were performed within one hour after tachyzoite collection from flasks.

2.2. Invasion rate (IR) and tachyzoite yield (TY) determination

IR was determined in a plaque assay where 100 purified tachyzoites of each isolate per well were added to MARC-145 monolayers in 24-well culture plates. Plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 72 h, and cell monolayers were then fixed with methanol at −20 °C for 30 min. and permeabilised with PBS-Triton X-100 (0.25%). The parasites were then labeled by immunofluorescence using anti-tachyzoite hyperimmune rabbit antiserum (1:4000) as the primary antibody (Alvarez-García et al., 2003) and a goat anti-rabbit conjugated to Alexa 594 (1:1000) (Molecular Probes, Eugene, OR, USA) as the secondary antibody. Plate wells were examined with a fluorescence-inverted microscope Nikon Eclipse E400 (Nikon Instruments Europe, Amsterdam, The Netherlands) at a magnification of 200× to count the labeled parasitophorous vacuoles and lysis plaques (events) per well after parasite growth. The invasion rate at 4 h (IR_{4h}) post-inoculation (p.i.) was determined as the number of events per well in cell monolayers washed three times with supplemented 2% FBS DMEM at 4 h p.i. (to remove all tachyzoites that did not invade at this time), and the total tachyzoite invasion rate (IR_T) was determined as the number of events per well in unwashed cell monolayers. Negative controls (the MARC-145 monolayer that was not inoculated) were included to eliminate potential fluorescent artifacts. No events were observed in the negative control samples.

The TY was determined by quantifying the number of tachyzoites at 56 h p.i. (TY_{56h}), prior to tachyzoite egress, by real-time PCR

Table 1
Summary of the name, host, geographical origin, passage number and pathogenic parameters evaluated in a pregnant BALB/c mouse model for the isolates included in this study (Dellarupe et al., 2014).

Isolate	Passage	Country	Reference	Dam morbidity*	DNA parasite detection in dams*	Pup mortality*,#	Vertical transmission rate*
Nc-Ger2	10–13[a]	Germany	Schares et al. (2005)	0/6 (0)	1/6 (16.7)	2/26 (7.7)	4/13 (30.8)
Nc-Ger3	13–15[a]	Germany	Schares et al. (2005)	0/4 (0)	2/4 (50)	4/21 (19)	1/11 (9.1)
Nc-Ger6	9–12	Germany	Schares et al. (2005)	0/5 (0)	2/5 (40)	1/25 (4)	3/13 (23.1)
Nc-6 Arg	12–14[a]	Argentina	Basso et al. (2001)	0/3 (0)	3/3 (100)	0/13 (0)	7/13 (53.8)
Nc-Bahia	15–17[b]	Brazil	Gondim et al. (2001)	3/4 (75)	4/4 (100)	15/15 (100)	8/8 (100)
Nc-Liv	15–18[a]	UK	Barber et al. (1995)	2/4 (50)	4/4 (100)	19/19 (100)	8/8 (100)

* Data obtained in a previous study (Dellarupe et al., 2014). The ratios indicate number of clinically affected or *N. caninum* ITS1-PCR-positive mice/the number of the analyzed mice. The number between brackets is the %.

\# All mice with clinical signs succumbed to infection at day 50 p.i. Pup morbidity and mortality were identical.

[a] Total passages after re-isolation in cell culture from inoculated mice.

[b] Numbers of passages in our laboratory from a cryovial of the Nc-Bahia isolate provided by L.F. Gondim.

(qPCR) using previously described primers and conditions (Collantes-Fernandez et al., 2002). In this assay, 2×10^5 purified *N. caninum* tachyzoites per well were added onto MARC-145 monolayers in 24-well culture plates and incubated as described above. The non-invading parasites were then removed by washing the monolayer at 4 h p.i. as described above. At 56 h p.i., the media were removed and the cell cultures and tachyzoites were recovered in 100 μL of PBS, 100 μL of lysis buffer and 10 μL of proteinase K. The samples were transferred to Eppendorf tubes and were stored at −80 °C prior to DNA extraction. Genomic DNA was extracted from cellular samples using a commercial kit (Real Pure DNA Extraction, Durviz, Spain) according to the manufacturer's instructions. Genomic DNA was eluted in a final volume of 50 μL. Each TY assay was performed in parallel plate duplicates. One plate remained unprocessed for DNA extraction and the other was used to monitor tachyzoite growth by microscopy. Photomicrographs were taken at 400× on an inverted microscope (Nikon Eclipse E400) connected to a digital camera.

The number of parasites and the amount of host cell DNA in the samples were measured in 5 μL of genomic DNA (20–50 ng/μL of DNA) from parasite cultures by real-time PCR targeting the Nc-5 region and the 28S gene, respectively (Collantes-Fernandez et al., 2002). Tachyzoite numbers were estimated by the interpolation of the *Ct* values on a standard curve performed with 10-fold serial dilutions of tachyzoite DNA (resembling 5×10^5–5×10^{1} tachyzoites DNA on each reaction) in a solution of 20 ng/μL of MARC-145 genomic DNA. The amount of host cell DNA for each sample was assessed by interpolation of the results of the *Ct* values on a standard curve performed with 200 ng, 100 ng, 40 ng, 20 ng, 5 ng and 1 ng of genomic DNA from MARC-145 cells. All samples for tachyzoite quantification were run in duplicate. Then, the tachyzoite number, determined by the median *Ct* value for each sample, was adjusted to the amount of 100 ng of host cell DNA as assessed by the 28S real-time PCR.

All the isolates were assayed in quadruplicate (four replicates) with IR and TY determined by at least two independent experiments. The Nc-Liv isolate was included as an inter-assay control in each batch of experiments. Significant differences were not found between IRs and TYs determined for the Nc-Liv inter-assay. Uninoculated MARC-145 monolayers were also included in both IR and TY assays to eliminate potential fluorescence artifacts in the immunofluorescence assays and as negative controls in each batch of PCRs, respectively.

2.3. Statistical analysis and correlation analysis

The differences between the IRs determined for the washed and unwashed batch of each isolate were compared using the Mann–Whitney *U* test (washed at 4 h p.i. *versus* unwashed). The Kruskal–Wallis test was employed for comparisons among the IRs (4 h p.i. and 72 h p.i.) and the TY_{56h}s displayed by the different isolates. When statistically significant differences were found with the Kruskal–Wallis test, Dunn's multiple-comparison test was then applied to examine all of the possible pairwise comparisons. Statistical analyses were carried out using a dataset composed of the values determined for each replicate obtained from independent experiments. The significance for these analyses was established at $P < 0.05$. The Spearman's rank correlation coefficient (ρ) was applied to investigate the potential association between the *in vitro* parameters evaluated in this study (the average of IR_{4h}, IR_T and TY_{56h}) and the dam morbidity, DNA parasite detection in dams, vertical transmission rate and pup mortality induced by these isolates in a BALB/c pregnant mouse model (Table 1) (Dellarupe et al., 2014). The threshold for significance in the correlation analysis was $P < 0.06$. Statistical and correlation analyses were performed, and graphics were generated using GraphPad Prism 5 Demo (v. 5.00 software Graph Pad).

3. Results

The IR_s of almost all isolates did not show differences in pairwise analyses between the IR_{4h} and IR_T ($P > 0.05$, U Mann–Whitney test). The only significant difference between the IR_{4h} and IR_T was found in the Nc-Ger3 isolate ($P = 0.0356$, U Mann–Whitney test). The IR_{4h} showed significant differences among the isolates studied ($P < 0.0001$, Kruskal–Wallis test). The Nc-Bahia demonstrated a higher IR_{4h} than the remaining groups, except the Nc-Liv isolate, which also demonstrated a significantly higher IR_{4h} than the Nc-Ger2 and Nc-Ger6 isolates (Dunn's test) (Fig. 1A). An analysis of the IR_T revealed similar differences among the groups ($P < 0.0001$, Kruskal–Wallis test), with a higher IR_T found for the Nc-Bahia isolate in comparison to the Nc-Ger2, Nc-Ger6 and Nc-6Arg isolates (Dunn's test). In addition, Nc-Ger3 and Nc-Liv were found to have a higher IR_T than the Nc-Ger2 and Nc-Ger6 (Dunn's test) (Fig. 1B).

A microscopic examination of inoculated cultures throughout the lytic cycle revealed that most of the isolates were located as tachyzoite pairs in vacuoles at 24 h p.i. (Fig. 2). Parasitophorous vacuoles (PVs) were also observed in Nc-Bahia and Nc-Liv cultures at 24 h p.i. as rosettes with a limited number of tachyzoites (4–16), suggesting that these isolates had begun their exponential tachyzoite growth phase at an earlier time point. At 48 h p.i. small PVs were observed in the Nc-Ger6 culture, whereas the remaining isolates presented medium-sized PVs (>16). At 72 h p.i., Nc-Ger6 had only developed small PVs, indicating a reduced growth rate for this isolate, whereas the Nc-Ger2, Nc-Ger3 and Nc-6Arg cultures

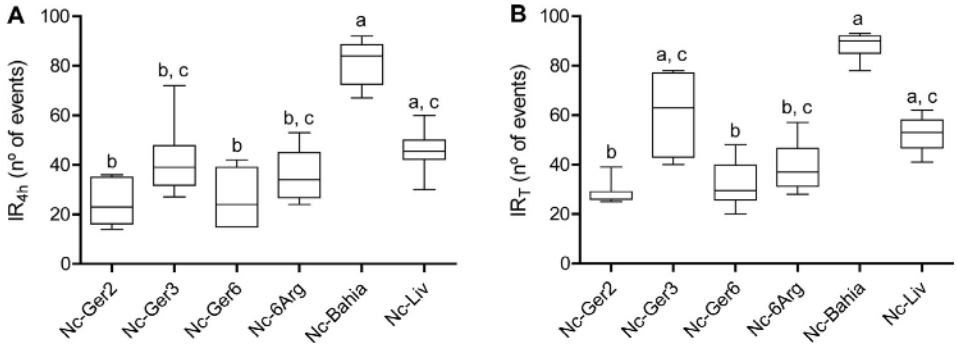

Fig. 1. Invasion rates (IRs) determined for each *N. caninum* isolate included in this study. Box-plot graphs representing the medians, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers) of IRs assessed *in vitro* in quadruplicate in two individual experiments for each *N. caninum* isolate at 4 h p.i. (IR_{4h}) (A) and at 72 h p.i. (IR_T) (B). [a,b,c]Different letters over the boxes indicate significant differences between the IR values of the *N. caninum* isolates according to the Kruskal–Wallis test followed by the Dunn's multiple-comparison test.

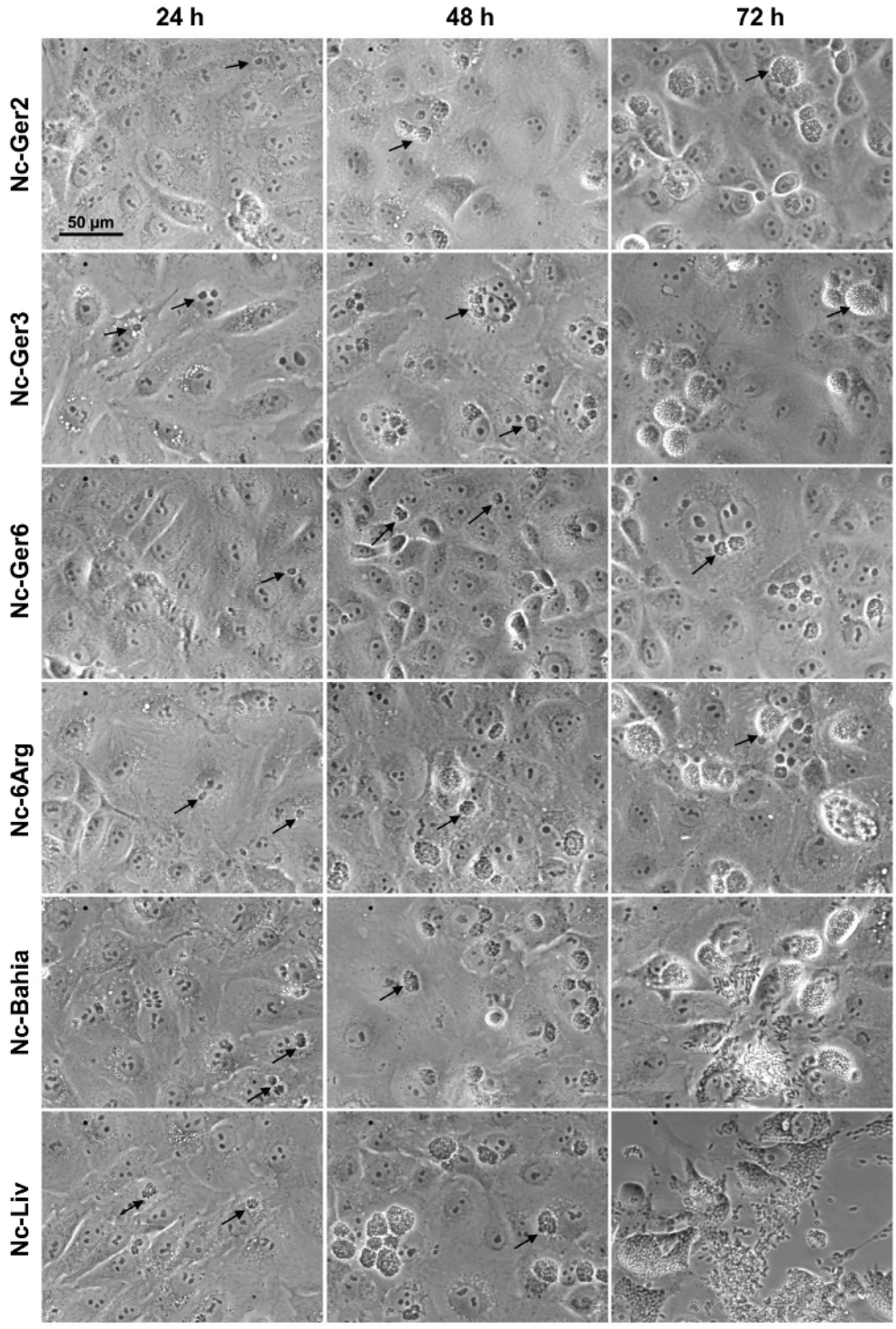

Fig. 2. Microscopic observations of inoculated cultures at 400×. At 24 h p.i., in most of the isolate cultures pairs of tachyzoites were visible (indicated by arrows). In addition to vacuoles with pairs, parasitophorous vacuoles (PVs) containing a limited number of tachyzoites (4–16) were observed in Nc-Bahia and Nc-Liv at 24 h p.i. At 48 h p.i., small PVs were observed in Nc-Ger6 isolate cultures, whereas the other isolates formed medium-size PVs (>16). At 72 h p.i. the Nc-Ger6 isolate cultures only contained small PVs, whereas the Nc-Ger2, Nc-Ger3 and Nc-6Arg exhibited large PVs and a limited number of lysis plaques indicating the recent egress of tachyzoites. The Nc-Bahia and Nc-Liv isolate cultures exhibited large PVs with a high number of lysis plaques where peripheral cells were re-invaded by tachyzoites and developed second-generation vacuoles. Arrows indicate tachyzoite pairs and parasitophorous vacuoles.

contained large PVs and a limited number of lysis plaques (as an indication of a recent tachyzoite egress). The Nc-Bahia and Nc-Liv cultures contained large PVs with a high number of lytic plaques formed by tachyzoites which had re-invaded cells peripheral to these plaques and developed second generation vacuoles. This suggests a high growth rate and an early tachyzoites egress in these isolates (Fig. 2).

The TY was assessed at 56 h p.i. when most of tachyzoites remained intracellular. Significant differences in the TY_{56h} values were also observed between the isolates ($P < 0.0001$, Kruskal–Wallis

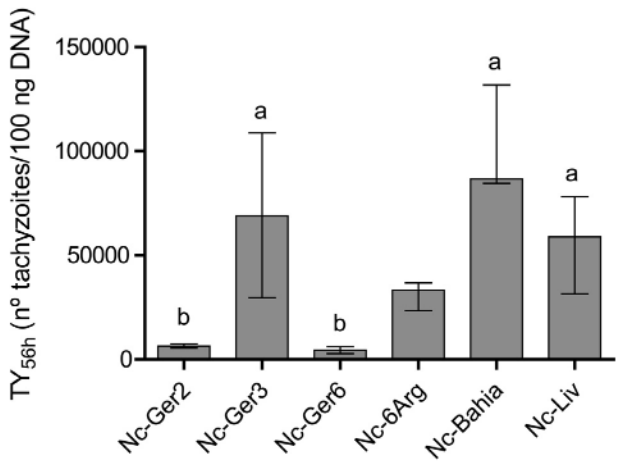

Fig. 3. The tachyzoite yield at 56 h p.i. (TY_{56h}) determined for each *N. caninum* isolate included in this study. A column-plot graph representing the medians and interquartile range (whiskers) of TY_{56h} assayed in quadruplicate for two individual experiments *in vitro* for each *N. caninum* isolate. [a,b]Different letters over columns indicate significant differences in TY values between *N. caninum* isolates according to the Kruskal–Wallis test followed by the Dunn's multiple-comparison test. Note that the absence of letters above columns indicates the absence of statistically significant differences between the indicated group and any other group.

Table 2
Spearman correlation analyses of the invasion rates at 4 h p.i. (IR_{4h}) and 72 h p.i. (IR_T) and the tachyzoite yield at 56 h pi (TY_{56h}), which were determined *in vitro* for each isolate in this study.

	IR_{4h}		IR_T		TY_{56h}	
	$\rho*$	P^*	$\rho*$	P^*	$\rho*$	P^*
Dam morbidity	0.8452	0.033	N.C.	–	0.8452	0.033
Parasite persistence in dams	0.8197	0.058	N.C.	–	N.C.	–
Neonatal mortality	N.C.	–	N.C.	–	0.8117	0.058
Vertical transmission rate	N.C.	–	N.C.	–	N.C.	–

$\rho*$ = Spearman rho coefficient.
P^* = P value (two-tailed).
N.C. = no correlation.

test). Nc-Ger3, Nc-Bahia and Nc-Liv exhibited the highest TY_{56h} compared to Nc-Ger2 and Nc-Ger6, which showed the lowest TY_{56h}s (by Dunn's test) (Fig. 3). Direct correlations were observed between the IR_{4h} with the TY_{56h} (Spearman's coefficient >0.9429, $P < 0.017$) and the IR_T with the TY_{56h} (Spearman's coefficient >0.885, $P < 0.033$).

Spearman correlation analyses were also applied to determine whether a potential association existed between the *in vitro* characteristics of isolates and their ability to be vertically transmitted and produce disease in mice as determined previously (Table 1). A significant correlation was found between IR_{4h} values with both morbidity and DNA parasite detection rate in dams (Table 2). Furthermore, TY_{56h} values significantly correlated with both dam morbidity and pup mortality (Table 2).

4. Discussion

N. caninum is an obligate intracellular parasite and the processes of tachyzoite invasion, namely, the adaptation to new cytoplasmatic conditions, intracellular proliferation, and the egress from host cells, are required for the maintenance and multiplication of the parasite *in vitro* and, more notably, for its *in vivo* dissemination within host tissues which is the cause of lesions and disease (Hemphill et al., 2006). The processes involved in the lytic cycle of the proliferative tachyzoite stage are therefore important for understanding the pathogenesis of neosporosis. Knowledge of essential elements involved in the lytic cycle may serve as a basis for the development of new therapeutic measures against neosporosis (Hemphill et al., 2013). Notably, a recent comparative analysis of the invasive and proliferative capacities of bovine *N. caninum* isolates obtained from asymptomatic calves revealed intra-species variability, which was associated with disease severity in a pregnant mouse model (Regidor-Cerrillo et al., 2011). *In vitro* invasion rate and tachyzoite yield were confirmed as traits associated with the virulence phenotype for these *N. caninum* isolates. In the present work, we compared a new population of canine *N. caninum* isolates *in vitro* and the results showed great similarities between canine and bovine isolates in terms of the dynamics of tachyzoite lytic cycle processes. Furthermore, marked differences in the IR and TY correlated with virulence observed in mice, confirming that the proliferative capacity may serve as a trait associated with the virulence phenotype also in isolates of canine origin as has been previously established for bovine isolates (Regidor-Cerrillo et al., 2011).

A similar time course for active tachyzoite invasion was found for most of these isolates, in which an insignificant increase was observed between the IR_{4h} and IR_T (at 72 h p.i.). This was likely to be a consequence of a loss of invasion capacity in tachyzoites which remained extracellular for more than 4 h, as previously reported for bovine isolates. Similarly, the maintenance of Nc-1 tachyzoites extracellularly at 37 °C for time periods longer than 4–6 h resulted in a significantly decreased parasite infectivity (Hemphill et al., 1996). Only the Nc-Ger3 isolate exhibited differences between IR_{4h} and IR_T, suggesting a longer time period during which Nc-Ger3 tachyzoites remain able to invade host cells after egress. An intracellular proliferation pattern similar to that of bovine isolates was also observed for canine isolates in this study (Regidor-Cerrillo et al., 2011). After invasion, tachyzoites did not multiply for a specific time period (lag phase) until initiating an exponential proliferation phase. We did not microscopically visualize parasitophorous vacuoles containing two or more tachyzoites until 24 h p.i., as this time period was likely necessary for tachyzoite adaptation to the intracellular niche and migration close to the cell nucleus before proliferation. As with bovine isolates, the time period of the lag phase may also vary among canine isolates because the visualization of small PVs at 24 h p.i. with Nc-Bahia and Nc-Liv indicated an earlier initiation of exponential growth for these isolates. The entry into the exponential growth phase was confirmed for all isolates by the visualization of mature PVs at 48 h p.i. This phase ended with the tachyzoites egress which was observed depending on the isolate between 56 h p.i.–72 h p.i. Interestingly although the proliferation rate was not determined in this study, a very long doubling time could be assumed for the Nc-Ger6 isolate by the visualization of small PVs and the absence of egress at 72 h p.i. Variations in the proliferation kinetics, including the proliferation rate and the time period of exponential proliferation, may define differences found in the TY. However, correlation analyses between IRs and TY_{56h} values indicate that the individual invasive capacities attained *in vitro* may be the major factor behind an isolate TY.

When the *in vitro* IRs and TY_{56h} values for each isolate were compared, significant differences were found among this population of canine isolates. As has been reported with bovine isolates, we observed that the canine isolates with the highest IRs and TY_{56h} (Nc-Bahia and Nc-Liv) caused the highest morbidity in dams, and the highest mortality and vertical transmission rate in neonates (100% succumbed to infection), whereas the isolates with the lowest IRs and TY_{56h} (Nc-Ger2 and Nc-Ger6) failed to induce morbidity in dams, and produced the lowest mortality and vertical transmission rate in neonates (Dellarupe et al., 2014). Correlation analyses of the above *in vitro* and *in vivo* parameters established a correlation of invasive capacities and TY with dam morbidity.

In addition, significant correlations between the TY_{56h} and pup mortality were observed in the progeny delivered from infected dams. Similar significant correlations had also been established for bovine isolates (Regidor-Cerrillo et al., 2011). These results support the hypothesis that isolates with the highest inherent proliferation capacities reach higher parasite burdens in target host-tissues (brain and placenta) contributing to host tissue damage and clinical signs. Moreover, highly proliferative isolates may easily evade the immune response mounted by the host. However, the Nc-Ger3 isolate, which exhibited high IRs and TYs likely due to its prolonged time of remaining infectious after inoculation into cell cultures, was moderately virulent for mice (Dellarupe et al., 2014). These findings suggest that the Nc-Ger3 infections, in contrast to infections with Nc-Liv and Nc-Bahia, may be effectively controlled in host tissues. It can be hypothesized that the Nc-Ger3 isolate induces a more effective Th1 immune response as detected previously *in vivo* (Dellarupe et al., 2014), which cannot be totally predicted under the conditions of the *in vitro* model. Even so, among the low-moderately virulent isolates, the Nc-Ger3 isolate caused the highest pup mortality and the IR and TY detected in the present study correlated with virulence observed in mice. However, although findings show evidence of variation in pathogenicity of *N. caninum* isolates in mice, extrapolation of results to the effect of a *N. caninum* infection in natural hosts must be done with caution (Dellarupe et al., 2014). Refinement and standardization of host-animal models of neosporosis would be helpful to understand the relevance of *N. caninum* diversity with regard to occurrence and severity of the disease in a natural host (Benavides et al., 2014; Regidor-Cerrillo et al., 2014).

Correlations of IRs and TY with vertical transmission rates were not detected in this study, likely due to other parasite traits including the dissemination capacity within host tissues and mechanisms supporting a transplacental dissemination which is the prerequisite for an infection of neonates (Collantes-Fernandez et al., 2012).

Although the basis for invasion and intracellular proliferation processes seems to be conserved among the Apicomplexa (i.e., the formation of a moving junction and secretion of the proteins from specialized organelles), other studies have revealed dissimilarities in host-cell interaction mechanisms and a divergence in the repertoire of molecular elements involved in host cell invasion between *N. caninum* and the closely related *T. gondii*, which may be the basis for differences in the host-range and pathogenicity among these species (Cowper et al., 2012; Reid et al., 2012). Significant variations in the abundance of proteins involved in tachyzoite invasion and proliferation among *N. caninum* isolates, as determined by proteomics, have also been described and may explain differences in proliferation and dissemination among these isolates (Regidor-Cerrillo et al., 2012). Nevertheless, the specific mechanisms involved in the divergences displayed by *N. caninum* isolates remain unclear and further studies are necessary to identify the key molecular elements that produce these specific tachyzoite processes in *N. caninum* as potential therapeutic targets.

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