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NcGRA2-RT-PCR to Detect Live Versus Dead Parasites in *Neospora caninum*-Infected Mice

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Abstract: In the present work, we optimized a recently established NcGRA2-RT-PCR based on RNA to detect live *Neospora caninum* parasites in tissue, and compared the results with the conventional inoculation of diagnostic specimen onto cell culture. C57BL/6 mice were experimentally infected with Nc-1 tachyzoites and subsequently euthanized 6 or 12 days post infection (dpi). Selected organs were used to search for parasites by (i) PCR using genomic DNA (gDNA), (ii) PCR using cDNA and (iii) *in vitro* inoculation of cell culture. At 6 dpi, *Neospora*-gDNA was detected in 34 out of 36 organs. Viable parasites were detected in 11 (NcGRA2-RT-PCR) and 15 (*in vitro* cultivation) out of 36 organs. Comparison of NcGRA2-RT-PCR and *in vitro* detection gave a fair agreement (kappa 0.29), whereas comparison of PCR using gDNA and RT-PCR or *in vitro* detection resulted in a slight agreement (kappa 0.05 and 0.08, respectively) only. At 12 dpi, parasite gDNA was found in 10 out of 36 organs. In 7 of these organs viability of parasites was confirmed with NcGRA2-RT-PCR and growth of parasites in cell culture. Comparison of NcGRA2-RT-PCR and *in vitro* detection gave a substantial agreement (kappa 0.8), whereas comparison of PCR using gDNA and RT-PCR or *in vitro* detection resulted in a moderate agreement (kappa 0.59 and 0.77, respectively). As NcGRA2-RT-PCR is almost as sensitive as *in vitro* cultivation in detecting live parasites, it represents a fast, easy and safe method of viable parasite detection, and thus an attractive alternative to the *in vitro* cultivation approach.

Keywords: *Neospora caninum*, NcGRA2-RT-PCR, *In vitro* inoculation.

INTRODUCTION

Currently, there is a generalized agreement on considering *Neospora caninum* as the protozoan pathogen which is most frequently associated with bovine abortion worldwide. The conventional diagnostic tools that have been made available so far, aimed at detecting the parasite and to discriminate *Neospora caninum* from infections with closely related parasites, such as *Toxoplasma gondii* or *Sarcocystis spp*.. Indirect techniques, based on serum antibody detection, prove parasite contact, but provide no information about the actual status of infection or disease, unless e.g. avidity is determined [1]. Direct detection techniques include histopathological investigations, complemented by molecular means such as the polymerase chain reaction (PCR) or by *in vitro* isolation of the parasite upon cell culture. Substantial efforts have been made to improve *N. caninum*-specific PCRs, which provide highly sensitive and specific parameters for the parasite detection by amplification of parasite specific sequences, such as the internal transcribed spacer 1 (ITS 1) [2] or a *Neospora*-specific genomic DNA (gDNA) sequence named Nc5 [3]. However, the presence of parasite gDNA in infected tissue does not necessarily provide information on the parasite viability, thus, conventionally, *in vivo* and *in vitro* tests are being used by inoculating appropriate samples into laboratory animals or cell culture [4]. Immunosuppressed mice, IFN-γ knock-out mice and gerbils (*Meriones unguiculatus*) are highly susceptible to *N. caninum* and were therefore used for corresponding tests [5-7]. The success of these methods strongly depends on the number of parasites and the state of the tissues. Especially in cell culture, opportunistic microbial contaminations are a big problem, as cultures have to be observed for a long time period.

Recently, we described a novel PCR that was established as a useful tool to distinguish between live and dead parasites in cell culture following a chemotherapeutic impairment of parasite viability [8]. NcGRA2-RT-PCR was specific for *N. caninum* and sensitive enough to detect 0.1 parasites equivalents per reaction [8]. In the present work, we compared the NcGRA2-RT-PCR for detection of live parasites *ex vivo* in organs from experimentally infected animals with the conventional approach to inoculate diagnostic material into appropriate cell culture subsequently maintained for 4 weeks.

MATERIALS AND METHODOLOGY

Tissue Culture Media, Chemicals and Drugs

If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Basel, Switzerland) and biochemical reagents were from Sigma (Buchs, Switzerland).

Tissue Culture and Parasite Purification

Cultures of Vero cells were maintained in RPMI 1640 medium (Gibco-BRL, Basel, Switzerland) supplemented with 5% fetal calf serum (FCS), 4 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B at 37 °C with 5% CO_2. Human foreskin fi-

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broblasts (HFF) were maintained in Dulbecco`s modified Eagle`s medium (DMEM) containing the same additives and were identically treated. Cultures were trypsinized at least once a week. *N. caninum* (Nc-1 isolate) tachyzoites were maintained in Vero cell monolayer cultures, during which time FCS was replaced with immunoglobulin G-free horse serum (HS). Tachyzoites were harvested when they were still intracellular by trypsinization of infected Vero cells followed by repeated passage through a 25-gauge needle. Host cell debris were removed from the parasites by separation on Sephadex-G25 columns as previously described [9]. The tachyzoites were counted using a Neubauer chamber and parasite numbers were adjusted by adding RPMI medium as appropriate for experimental infection.

Mice, Infection, Euthanasia and Sample Collection

12 wild-type C57BL/6 female mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and randomly separated into two groups with 6 mice each. Food mix and water were provided *ad libitum*. All mice (10 weeks of age) were infected intraperitoneally (i.p.) with 1 x 10^6 live Nc-1 tachyzoites suspended in 200 µl RPMI medium at the same time point. Mice were daily checked for clinical signs. A group of 6 mice was euthanized by CO_2-inhalation 6 and 12 days post infection (dpi). From all animals, a brain hemisphere, the lungs, heart, liver, kidney and uterus were removed, disrupted with a scalpel blade and subsequently divided into three parts. Two parts were immediately frozen in liquid nitrogen and stored at -80 °C prior to gDNA and RNA isolation. A third part was suspended in 1 ml RPMI medium and transferred into a falcon tube. The same volume of trypsin (0.05 % trypsin, 0.53 mM EDTA•4Na in hanks' balanced salt solution, Gibco-BRL) was added and the suspension was further disrupted with a syringe and needle starting from G18 to G23. The suspension was sedimented at 400 g to remove trypsin, and the remaining pellet was re-suspended in 4 ml DMEM medium containing 500 U/ml penicillin G, 500 µg/ml streptomycin, 1.25 µg/ml amphotericin B and 5% HS. Suspension was immediately used for inoculation of cell cultures.

Inoculation of Cell Cultures

HFF, as susceptible host cells, were grown to confluent monolayer in 24-well tissue culture plates, and subsequently inoculated with 1 ml of the diagnostic cell suspension per well. For each organ, 4 wells were inoculated with organ homogenates. The culture medium was changed after 24 h and then every second day. The cultures were daily checked for putatively growing parasites or contaminations. Cell cultures containing visible parasites and cultures grown for 4 weeks without visible parasites were lysed directly in the wells using 200 µl PBS containing Proteinase K (12 mAU/ml) and 200 µl AL buffer (DNeasy® blood and tissue kit; QIAGEN, Basel, Switzerland). Lysates were frozen at -80 °C prior to DNA purification.

DNA Extraction

DNA purification from organs was performed using the DNeasy® blood and tissue kit (QIAGEN) according to the standard protocol suitable for animal tissues. Frozen organs were equilibrated to room temperature and lysed overnight at 56°C in ATL buffer containing Proteinase K (12 mAU/ml). DNA purification from cell lysates was performed using the DNeasy® blood and tissue kit (QIAGEN) according to the standard protocol suitable for animal blood or cells. Frozen lysates were equilibrated to room temperature before starting. gDNA was eluted in 100 µl AE buffer, boiled at 95 °C for 3 min and frozen at -80 °C prior to PCR.

RNA Extraction

Total RNA isolation from brains was performed using the RNeasy® lipid tissue mini kit (QIAGEN) according to the standard protocol suitable for lipid animal tissues. Total RNA isolation from hearts and uteri was performed using the RNeasy® mini kit (QIAGEN) according to the standard protocol suitable for heart, muscle and skin tissues. Total RNA isolation from lungs, livers and kidneys was performed using the RNeasy® mini kit (QIAGEN) according to the standard protocol suitable for animal tissues. All tissues were disrupted in the corresponding buffer with a small mortar. Except of the brain lysates, all lysates were loaded onto QI-Ashredder™ spin columns (QIAGEN) and centrifuged at maximum speed for 2 min after disruption. RNA purification included a DNA digestion step with RNase-free DNase (QIAGEN) for 1 h at room temperature. RNA was eluted in 60 µl RNase-free water, boiled at 95 °C for 3 min and immediately used for cDNA synthesis.

Reverse Transcription

RNA concentration was measured with the NanoDrop (Thermo Scientific, Delaware, US) system. cDNA synthesis was performed using the Omniscript® Reverse Transcription kit (QIAGEN) according to the standard protocol for first-strand cDNA synthesis. Briefly, 0.5 µg random Primer (Promega, Wallisellen, Switzerland) and 100 ng (uterus), 500 ng (lung, kidney, heart) or 1000 ng (brain, liver) of RNA were used in a final volume of 20 µl reaction mix and incubated for 1 h at 37 °C. cDNA was boiled at 95 °C for 3 min and frozen at -80 °C prior to PCR.

Conventional PCR

Detection of parasite-specific gDNA by Nc5-PCR was done as previously described [10] with *N. caninum*-specific primers Np21plus and Np6plus in a thermal cycler (Gene Amp PCR System model 9700; Applied Biosystems, Basel Switzerland). For the mix, 20 pmol of each primer and 1 µl DNA in a final volume of 25 µl were used.

Detection of parasite-specific cDNA by NcGRA2-RT-PCR was done as recently described [8] with *N. caninum*-specific primers NcGRA2-F1 and NcGRA2-R2. PCR was performed using 25 pmol of each primer and 1 µl cDNA in a final volume of 25 µl.

Both PCR-mixes were performed using the AmpliTaq® DNA polymerase kit (Applied Biosystems). To prevent carry-over contamination from previous reactions, the samples were incubated with uracyl DNA glycosylase (UDG; Roche Diagnostics, Basel, Switzerland) for 10 min at 20 °C. The UDG was inactivated by incubation at 95 °C for 2 min. Each run included a negative (water) and a positive (purified parasites) sample.

Statistical Analyses

To measure the agreement between the methods, kappa statistic was used. Calculation and interpretation of kappa was performed as described by [11].

RESULTS

At 6 dpi, all of the 6 experimentally infected mice were devoid of any clinical signs. Following sacrifice, organs were removed from animals to extract gDNA and RNA, and to generate tissue cell suspension for inoculation onto host cell culture. Parasite gDNA was detected in 5 out of 6 brains, 6 out of 6 lungs, 6 out of 6 hearts, 5 out of 6 livers, 6 out of 6 kidneys and 6 out of 6 uteri from the group of experimentally infected animals. With the NcGRA2-RT-PCR, live parasites were detected in 2 out of 6 brains, 6 out of 6 lungs and 3 out of 6 hearts. NcGRA2-RT-PCR remained negative for all livers, kidneys and uteri of the same group of experimentally infected animals. Parasite proliferation was observed in HFF host cell culture inoculated with brains (2 out of 6), lungs (5 out of 6), hearts (1 out of 6), livers (2 out of 6), kidneys (1 out of 6) and uteri (4 out of 6) (Table **1**). Parasites started to visibly (microscopy) grow in culture between 14 and 29 days after inoculation.

With *Neospora*-PCR using gDNA 34 out of 36 tested organs were *Neospora*-positive. In 7 out of 34 cases, we could prove with both, NCGRA2-RT-PCR and *in vitro* cultivation, respectively, that parasites detected with conventional gDNA-PCR, actually were viable. In 15 organs, *Neospora*-gDNA was detected but viability of parasites could not be confirmed, neither by NcGRA2-RT-PCR nor by *in vitro* cultivation. Two organs were *N. caninum*-negative by all three methods. For 4 *Neospora*-gDNA positive organs, parasite viability was proven with NcGRA2RT-PCR only, whereas in another 8 organs parasite viability was shown in cell culture only. Comparison of viable parasite detection using RT-PCR or *in vitro* detection resulted in a fair agreement (kappa 0.29) with an observed agreement of 67% and an expected agreement of 53%. Slight agreements were observed when results of PCR using gDNA were compared with RT-PCR and *in vitro* detection (kappa 0.05 and 0.08, respectively).

By day 12 dpi, none of the infected mice showed any clinical signs due to neosporosis. All 6 mice were euthanized and organs were taken for gDNA and RNA extraction, and to obtain an organ cell suspension for subsequent diagnostic inoculation in host cell culture. Parasite gDNA was detected in 6 out of 6 brains, 1 out of 6 lungs and 3 out of 6 uteri, whereas no gDNA was found in hearts, livers and kidneys. With the NcGRA2-RT-PCR, live parasites were detected in 5 out of 6 brains, whereas all the other organs remained respectively negative. Upon *in vitro* cultivation, parasite proliferation was observed in host cell culture inoculated with brains (6 out of 6) and in 1 out of 6 uteri. No parasites were seen after 4 weeks in cultures inoculated with lungs, heart, livers and kidneys (Table **2**). After inoculation in host cell culture, parasites started to grow between days 8 and 17.

In 5 *Neospora*-gDNA positive brains, parasite viability was observed by both, NCGRA2-RT-PCR and *in vitro* cultivation. For 2 *Neospora*-gDNA positive organs, we demonstrated parasite viability upon cell culture approach only. In 3 organs, although *Neospora*-gDNA was detected, a respective viability of parasites could not be confirmed, neither by NcGRA2-RT-PCR nor by *in vitro* cultivation. Furthermore, 26 out of 36 organs were negative for all three diagnostic methods.

Comparing RT-PCR and *in vitro* detection results, we observed an agreement of 94%. The agreement by chance was 72%. Kappa calculation gave a substantial agreement (kappa 0.8). PCR using gDNA compared with RT-PCR and *in vitro* detection resulted in a moderate agreement (kappa 0.59 and 0.77, respectively).

DISCUSSION AND CONCLUSION

The availability of routine diagnostic tools to detect *N. caninum*-infections in animals prompted a more detailed approach, aimed at discriminating between alive and died-out parasites. Conventional tools such as serology, histopathology and PCR yield an etiological diagnosis, but no direct information on actual viability of parasites at the time point of collection of diagnostic samples. The only alternative so far consisted in diagnostic *in vivo* or *in vitro* cultivation of sample suspensions by using susceptible mice or cell culture.

Table 1. ***Neospora caninum*** **detection 6 days post infection**

	Mouse 1			**Mouse 2**			**Mouse 3**			**Mouse 4**			**Mouse 5**			**Mouse 6**		
	gDNA	**cDNA**	***in vitro***	**gDNA**	**cDNA**	***in vitro***	**gDNA**	**cDNA**	***in vitro***	**gDNA**	**cDNA**	***in vitro***	**gDNA**	**cDNA**	***in vitro***	**gDNA**	**cDNA**	***in vitro***
Brain	**+**	**+**	**+**	+	-	-	€	€	€	+	-	-	+	-	+	+	+	-
Lung	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	+	+	-
Heart	**+**	**+**	**+**	+	-	-	+	+	-	+	+	-	+	-	-	+	-	-
Liver	+	-	-	+	-	+	+	-	-	+	-	+	+	-	-	€	€	€
Kidne	+	-	-	+	-	-	+	-	-	+	-	+	+	-	-	+	-	-
Uterus	+	-	-	+	-	+	+	-	-	+	-	+	+	-	+	+	-	+

Neospora caninum detection 6 days post infection.

Neospora caninum was detected by three different methods (PCR based on gDNA or cDNA and *in vitro* cultivation) in a group of six mice experimentally infected with *N. caninum* tachyzoites. Animals were investigated 6 days post infection.

€ all detection methods negative.

+ all detection methods positive.

+ positive result.

- negative result.

Table 2. ***Neospora caninum*** **Detection 12 Days Post Infection**

	Mouse 7			Mouse 8			Mouse 9			Mouse 10			Mouse 11			Mouse 12		
	gDNA	cDNA	*in vitro*	gDNA	cDNA	*in vitro*	gDNA	cDNA	*in vitro*	gDNA	cDNA	*in vitro*	gDNA	cDNA	*in vitro*	gDNA	cDNA	*in vitro*
Brain	+	-	+	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Lung	€	€	€	+	-	-	€	€	€	€	€	€	€	€	€	€	€	€
Heart	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€
Liver	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€
Kidney	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€	€
Uterus	€	€	€	+	-	-	+	-	-	+	-	+	€	€	€	€	€	€

Neospora caninum was detected by three different methods (PCR based on gDNA or cDNA and *in vitro* cultivation) in a group of six mice experimentally infected with *N. caninum* tachyzoites. Animals were investigated 12 days post infection.
€ all detection methods negative.
+ all detection methods positive.
+ positive result.
- negative result.

Such techniques may be valuable for research purposes, but for routine or mass investigations, they are tedious, time-consuming and expensive. Nevertheless, distinction between live and dead organisms is an important tool, for example, in testing chemotherapeutical efficacy or in assessing infectiosity of carrier animals or organs. Recently, a RT-PCR based on the *NcGRA2* gene was established in view to distinguish between parasiticidal and parasitostatic efficacy of given compounds in cell-culture-based assays [8].

In the present work, we used the same NcGRA2-RT-PCR to detect live parasites in organs obtained from experimentally *Neospora*-infected mice. To validate the results, we carried out parallel experiments by inoculating identical diagnostic materials into appropriate cell culture. In previous works, it was shown that at an early stage of infection, *N. caninum* can be detected in almost all organs of infected animals [12]. In the present study, mice examined at 6 dpi yielded similar findings, in that we revealed the presence of parasite-gDNA in almost all organs. Nevertheless, DNA does not automatically imply viable parasites, whereas RNA synthesis takes place only in live organisms. In the present study, only 19 out of 34 DNA-positive organs actually contained viable parasites as detected with NcGRA2-RT-PCR or *in vitro* cultivation or both, resulting in slight agreement only between DNA and viable parasite detection. During the course of infection, parasites may die due to host immune reactions or by other reasons, and DNA may still be present in affected organs, conversely to RNA that is much less stable. From 19 samples with live parasites, viable parasites were detected in 7 organs with both methods, NcGRA2-RT-PCR and *in vitro* cultivation. Here, lungs were the organs with the highest agreement. For other organs, either RT-PCR or *in vitro* cultivation gave a positive result, due to the fact that only few parasites were available in the corresponding organ and an unequal distribution during the experimental procedure. Nevertheless, results of RT-PCR and *in vitro* cultivation showed a fair agreement.

After the acute phase of infection, reflected by the presence of parasites in multiple organs, tachyzoites withdraw from most organs to switch into the brain, putatively related to the fact that this organ is less involved in systemic immune reactivity. In our experiment, at day 12 dpi, we preferentially found parasite-gDNA in the brain. Except of one brain, we could confirm with both viability tests that the gDNA belonged to live parasites. Substantial agreement was observed between RT-PCR and *in vitro* cultivation approach. Both viability tests never yielded a positive result when the PCR using gDNA was negative.

In comparison, NcGRA2-RT-PCR is as sensitive as *in vitro* detection of viable parasites, but RT-PCR is much faster and easier to handle than the *in vitro* cultivation. Especially for mass investigations, including a high number of tissue samples, RNA isolation and RT-PCR presents a fast method to detect remaining live parasites. Furthermore, there is less risk of sample contamination, especially if the PCR is done with material directly and freshly isolated from mouse tissue, this compared to culturing the materials for 4 weeks. The NcGRA2-RT-PCR appeared as a useful alternative tool to determine viability status of *N. caninum* in organs of infected animals. Distinction between live and dead parasites is important in treatment and vaccination studies, and RT-PCR provided a fast, easy and safe option for this purpose. The use of immunocompromised animals to detect viable parasites is the most sensitive technique so far. However, as detection of viable parasites in tissue from infected animals by NcGRA2-RT-PCR was tested for the first time, we used *in vitro* cultivation – in reference to [8] - to obtain information about sensitivity and handling procedure of the new technique. In a further future step, NcGRA2-RT-PCR will be compared with inoculation of diagnostic materials (obtained from naturally infected bovines) into immunocompromised mice.

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