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In vitro culture combined with quantitative TaqMan PCR for the assessment of *Toxoplasma gondii* tissue cyst viability

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ABSTRACT

Toxoplasma gondii is a serious food-borne pathogen with a worldwide distribution. In order to assess the risk of contracting toxoplasmosis from certain foods, many studies rely on the molecular detection of *T. gondii* DNA. However, determining the viability of parasites in positive samples is much more problematic. In this paper we describe a novel viability assay that relies on semi-quantitative comparison of the amount of parasite DNA present in samples used to infect host cell monolayers *in vitro*, and the amount of DNA detected in the same monolayers after 23 days incubation. Our assay is robust, easy to perform and interpret and offers a viable alternative to bioassays, for use in epidemiological studies, or the evaluation of specific food safety treatments.

1. Introduction

Toxoplasma gondii is an intracellular sporozoan parasite that can cause serious disease in both humans and domestic and wild animals. It is estimated that about one-third of the human population worldwide has been exposed to this parasite (Hill and Dubey, 2002). Congenital toxoplasmosis may result in abortion, birth defects, mental retardation or blindness. In immunocompromised people infections are characterised by encephalitis and disseminated toxoplasmosis and may become life-threatening (Tenter et al., 2000). Moreover, the long-held view that in healthy adults infections are subclinical, is now being challenged as more cases of serious toxoplasmosis in immunocompetent patients are being reported (reviewed by McAllister, 2005). These infections may manifest as psychiatric disorders such as schizophrenia, lymphadenopathy, fever, debilitation, ophthalmitis, or severe multisystemic disorders.

Humans can contract toxoplasmosis either by congenital transmission, by ingestion of food and water contaminated with oocysts shed in the faeces of infected cats (the final hosts of *T. gondii*), or by ingestion of tissue cysts in raw or undercooked infected meat. The latter route of transmission, particularly the consumption of undercooked or cured lamb, pork or game is considered the most important risk factor for contracting toxoplasmosis in Europe (Cook et al., 2000).

Over recent years a number of epidemiological studies have been undertaken to determine the prevalence of *T. gondii* in certain meats and assess the risk to the consumer (Warnekulasuriya et al., 1998; Wyss et al., 2000; Aspinall et al., 2002). However, while the detection of parasite DNA in meat samples is straight-forward, assessment of the viability of *Toxoplasma* tissue cysts is challenging. To address this problem we have developed a new robust method for the assessment of *Toxoplasma* tissue cyst viability that combines an *in vitro* culture approach with quantitative PCR.

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2. Materials and methods

2.1. Optimisation of in vitro culture methods for T. gondii

A culture-adapted *T. gondii* strain (S48) was kindly provided by L. Innes, Moredun Research Institute, Scotland. The parasites were maintained in continuous culture in VERO cells following published procedures (Hughes et al., 1986). Briefly, VERO cells were maintained in IMDM medium (with 25 m HEPES, Bio-Sciences, Ireland) supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamine, penicillin (0.05 mg/ml) and streptomycin (0.05 mg/ml) and incubated at 37 °C at 5% CO_2. Once they had reached confluence (after 3–4 days), the VERO cells were subcultured by trypsinisation in 0.25% warm trypsin-EDTA (Bio-Sciences, Ireland). Following washing in fresh culture media, viable cells (i.e. cells excluding trypan blue stain) were counted in a haemocytometer and used to initiate new VERO cell cultures. For parasite maintenance, VERO cell monolayers were infected with tachyzoites at a 2 tachyzoites: 1 VERO cell ratio. After 3–4 days, or once the majority of infected host cells had detached, the remaining attached cells were scraped from the culture flask, concentrated by centrifugation ($850 \times g$, 10 min) and used to infect new monolayers.

In an attempt to optimise the parasite growth rate, IMDM medium was replaced with MEM (with Earle's salts and 25 mM HEPES, Bio-Sciences, Ireland) and HL-1 medium (BioWhittaker, Lonza, Belgium). All media were supplemented with either 5% FCS (Bio-Sciences, Ireland), 5% horse serum (Sigma–Aldrich, Ireland) or 5% rabbit serum (Sigma–Aldrich, Ireland) in addition to 2 mM L-glutamine, penicillin (0.05 mg/ml) and streptomycin (0.05 mg/ml). All optimisation experiments were carried out in 25 cm^2 tissue culture flasks with seeding concentrations of 1×10^5 VERO cells infected with 2×10^5 tachyzoites the following day. Each medium/serum concentration was tested through at least four subcultures in single (VERO cells) or duplicate (tachyzoites) flasks. VERO cell and parasite growth was assessed by five separate counts using a haemocytometer as described above. Cell concentrations were analysed using the nonparametric Kruskal–Wallis H-test (McClave et al., 1997) followed by the Wicoxon signed rank test to determine differences between individual groups (Data desk 6.2). The null-hypothesis was rejected at $P < 0.05$.

2.2. Initiation of T. gondii in vitro cultures using tissue cysts

2.2.1. T. gondii-infected tissue samples

Four pregnant ewes were each administered 3000 sporulated *T. gondii* ocysts (strain M4) per os on day 90 of gestation. The ewes were euthanased 28 days post challenge and, in total, 45 placentomes (between 9 and 13 placentomes per animal) were dissected from the placenta post mortem. The experimental infection was conducted under licence from the Department of Health and Children and received approval from an in-house Ethical Review Panel. The oocysts were kindly provided by D. Buxton, Moredun Research Institute.

2.2.2. Transfer into in vitro culture

The methods used to initiate *T. gondii* cultures in VERO cells were essentially those published by Miller et al. (2001). Immediately prior to culture initiation, fresh or stored vacuum-packed placentomes were surface-sterilised by submersion in 70% ethanol, followed by 2 washes in PBS supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml). Using a sterile scalpel and forceps, approx half of the placentome (4–5 g) was homogenised and then incubated in 10 ml warm trypsin-EDTA (0.05%, Bio-Sciences, Ireland) containing penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml) at 37 °C with shaking. After 1 h the digest was centrifuged ($1500 \times g$, 10 min), and the pellets transferred into 25 cm^2 tissue culture flasks that had been seeded with VERO cells the previous day. After a 2 h incubation at 37 °C and 5% CO_2, the digest was poured off, centrifuged and the pellet retained for DNA extraction. The infected monolayer was overlaid with warm HL-1 medium, supplemented with 5% FCS, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml). The following day the supernatant was replaced again with fresh medium and 1×10^5 VERO cells were added to flasks that showed no attached VERO cells. Subsequently the culture medium was changed every 5 days. After 23 days the monolayers were scraped off the flasks, the cell suspension centrifuged ($1500 \times g$, 10 min), and the whole pellet used for DNA extraction. The 23 day cut-off was chosen because previous *in vitro* isolation experiments had shown that if parasites could be detected microscopically they were usually apparent between days 17 and 21 after culture initiation (data not shown).

Cultures were initiated from placentomes on the day the animals were slaughtered, and then on days 1, 2, 3, 4, 6, 8, 11 and 13 after. All placentomes that were not immediately used were individually vacuum-packed and stored at 4 °C.

2.3. Molecular analysis and quantification

2.3.1. DNA extraction

Using the High Pure PCR Template Preparation kit (Roche) DNA was extracted from the material used to inoculate the flasks and from the infected monolayers harvested after 23 days. The manufacturer's protocol for tissue samples was followed except that instead of 50 μg of tissue, approx 200 μg of the pellet used to inoculate the VERO cell cultures or the entire cell pellet harvested after 23 days in culture (approx 200 μl cell suspension) were added in the first step. In the final step, DNA was eluted into 100 μl instead of 200 μl elution buffer.

2.3.2. Conventional nested PCR

All pellets of trypsinised placentome samples used to infect VERO cell monolayers were analysed for the presence of *T. gondii* using conventional nested PCR. The PCR protocol targeted a 302 bp fragment of the ITS 1 region (Jauregui et al., 2001). For the primary PCR we used primers developed by Hurtado et al. (2001). In brief, the primers were 5'-CCT TTG AAT CCC AAG CAA AAC ATG AG-3'