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Institute of Parasitology
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Combination of cell culture and quantitative PCR (cc-qPCR) for assessment of efficacy of drugs and disinfectants against *Cryptosporidium parvum*

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Dedicated to departed soul of my mother

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List of abbreviations

bp = base pairs
Ca^{2+} = Calcium
cc-qPCR = cell culture and quantitative PCR
CO_2 = carbon dioxide
Ct = cycle threshold
CV = coefficient of variation
ddH_2O = double distilled water
DMSO = Dimethyl sulfoxide
DNA = deoxyribo nucleic acid
dNTP = Deoxyribonucleotide triphosphate
DVG = Deutsche Veterinärmedizinische Gesellschaft (German Veterinary Society)
EC_{50} = Effective concentration 50%
fig = figure
g = acceleration of gravity
h = hour
h p.i. = hour post inoculation
HCl = Hydrochloric acid
HCT-8 = Human colonic tumor (Human ileocecal adenocarcinoma) cells
hsp 70 = heat shock protein 70
kDa = kilo Dalton
µg = microgram
µl = microliter
µM = micromolar
M = molar
mg = miligram
Mg^{2+} = magnesium
$MgCl_2$ = magnesium chloride
min = minute
ml = millilitre
mM = milimolar
MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide;
(Methyl- Thaizoleblau, Thaizolylblau, Tetrazolium bromide)
ng = nanogram
nm = nanometer (wavelength)
nM= nanomolar
P = statistical probability value
PBS = phosphate buffered saline
qPCR = quantitative polymerase chain reaction
r^2 = statistical linear correlation
ROS = reactive oxygen species
RPMI 1640 = Roswell Park Memorial Institute 1640
s = second
SD = standard deviation
spp = species

1 Introduction

Cryptosporidium parvum is one of the most common and significant enteric pathogens among the apicomplexa, a phylum that includes also other important pathogens such as *Toxoplasma*, *Isospora, Eimeria* and *Plasmodium* species (GOODGAME 1996). The parasite normally causes self limiting diarrhoea in the immune host but disease may display particularly severe in neonatal and immunocompromised mammals (DUBEY et al. 1990, O'DONOGHUE 1995) resulting in economic loss in farm animals (DE GRAAF et al. 1999). Infected animals shed oocysts (CURRENT 1985) with faeces and these may be transmitted to a variety of hosts including man through contaminated water and food (CASEMORE et al. 1997, SMITH and ROSE 1998, DAWSON 2005, SMITH et al. 2007), or by direct contact with the infected host (PREISER et al. 2003, KIANG et al. 2006). The tiny size and robustness of *Cryptosporidium* oocysts, high oocyst resistance to chemicals and environmental conditions, and the low infective dose are features of *C. parvum* that play a role in transmission and epidemiology of this parasite (CAREY et al. 2004).

Presently no immunization or satisfactory specific therapeutic options are available for the control of cryptosporidiosis (DAS 1996, RAMRATNAM and FLANIGAN 1997, LAING 1999). Moreover, the parasite is resistant to most anticoccidal drugs (SMITH and CORCORAN 2004, RAMIREZ et al. 2004) and many disinfectants (O'DONOGHUE 1995, BARBEE et al. 1999). Potent agents are urgently required for controlling cryptosporidiosis. Curcumin (diferuloylmethane, principal colouring agent of the herb *Curcuma longa*) is a natural polyphenolic compound reported to have antiprotozoal activities against *Plasmodium falciparum* (CUI et al. 2007), *Leishmania* spp. (KOIDE et al. 2002, DAS et al. 2008), *Trypanosoma* spp. (NOSE et al. 1998), and *Giardia lamblia* trophozoites (PEREZ-ARRIAGA et al. 2006).

Reliable and reproducible *in vivo* or *in vitro* models are required for development and evaluation of new potent drugs and disinfectants. The mouse model has frequently been used to determine *C. parvum* oocyst viability after drug application and disinfection measures (KORICH et al. 1990, FINCH et al. 1993, ARROWOOD et al. 1996, NEUMANN et al. 2000, KAYSER et al. 2002, CASTRO-HERMIDA and ARES-MAZAS 2003, MELE et al. 2003). A chicken infection model for *Eimeria tenella* oocysts was standardized for routine screening of anticoccidial disinfectants (DAUGSCHIES et al. 2002) and is officially approved by the German Veterinary Society (DVG).

However, animal models are laborious, expensive, ethically concerned and quantitative results are less easily obtained than in *in vitro* models (CAMPBELL et al. 1982). For evaluation of drug effects against *C. parvum* and reliable estimation of infectivity of oocysts *in vitro*, the parasite must be cultured in human or animal cells. *C. parvum* demonstrates many developmental attributes common to enteric coccidia (O'DONOGHUE 1995). Coccidia development in cultured cells, ideally complete development *in vitro*, has been reported for only a few species prior to 1991. Comparison of various cell lines revealed that HCT-8 (Human ileocecal adenocarcinoma) cells best supported the life cycle of *C. parvum* (UPTON et al. 1994) suitably reflecting the ability of oocysts to cause infection of animals (MELONI and THOMPSON 1996, SHIN et al. 2001, JENKINS et al. 2003). Cell culture is successfully used to evaluate *C. parvum* infectivity and for quantitatively estimating inactivation of oocysts by disinfectants (SLIFKO et al. 1999, 2002). The methods developed for oocyst infectivity analysis in cell culture are similarly applied for assessing growth inhibition of *Cryptosporidium* by anticryptosporidial compounds. Methods for quantitative analysis of cell culture infection include enzyme-linked immunosorbent assay (WOODS et al. 1995), immunofluorescence microscopy (SLIFKO et al. 1997), colorimetric *in situ* hybridization (ROCHELLE et al. 2002) and reverse transcription (ROCHELLE et al. 1997). In comparison to other methods real time PCR is suitable for detection as well as accurate quantification of DNA and thus parasite numbers (HEID et al. 1996). It is reported as a fast analytical procedure for detection and quantification of *Cryptosporidium* in cell culture (MACDONALD et al. 2002, DI GIOVANNI and LECHEVALLIER 2005).

We report here a combination of cell culture and quantitative PCR (cc-qPCR) suited to easily, reliably and reproducibly quantify reproduction of *C. parvum* for routine screening of pharmaceutical compounds and anticoccidial disinfectants under standardized conditions. The aims of the current studies were:

1) To establish a protocol combining cell culture and qPCR (cc-qPCR) for reliable assessment of infectivity of *C. parvum* oocysts
2) To analyse the potential of curcumin for control of *C. parvum* in this model
3) To assess whether cc-qPCR may be proposed to replace the chicken infection model for routine testing of disinfectants.

2 Overview of the scientific works

C. parvum was maintained in the laboratory through passage twice in a year by oral infection of a day old calf with oocysts (9 x 10^6). Oocysts were isolated from faeces by ether sedimentation and saturated sodium chloride flotation techniques. The cleaned oocysts were stored at 4°C in phosphate buffered saline (PBS, pH 7.4) supplemented with streptomycin (200 µg/ml) and amphotericin B (5 µg/ml). Oocysts used for drug screening were stored for up to 9 months and for up to 3 months for inactivation assays. The excystation rates of the oocysts ranged from 56 to 82%. HCT-8 cells were maintained for cell culture assays and cells of less than 19 passages were used in these experiments.

The cell culture infection assay was standardized for reliable and reproducible estimation of parasite multiplication by real time quantitative PCR (qPCR). DNA was extracted from samples using QIAamp mini kit (Qiagen, Hilden, Germany) and stored at -20° C if not used immediately. Each reaction mixture of 25 µl contained 1x PCR buffer (ABgene mastermix, AB gene limited, Surrey, England) supplemented with dNTP, $MgCl_2$ and Taq-polymerase, 300 nM forward primer and 900 nM reverse primer (each 25 µM, Biopolymer, Ulm, Germany), 30 nM of ROX reference dye (1 mM AB gene) and 200 nM of TaqMan Probe (25 µM, Biopolymer, Ulm, Germany). The reaction was performed in a Mx3000P® qPCR system (Stratagene, La Jolla, USA) with the condition of enzyme activation at 95°C for 15 min, 40 cycles of denaturation at 94-95°C for 15 s, and finally annealing and elongation at 60°C for 1 min. Each reaction was run in triplicate. The target sequence of 70 kDa heat shock protein (hps70) gene (single copy) of *C. parvum* was amplified by qPCR and reported as a cycle threshold (Ct) value of fluorescence signal for each sample. A standard curve was run in each experiment. By comparing the Ct values of unknown samples with the Ct values of the standard curve, the starting copy numbers (one copy gene corresponding to one sporozoite) of nucleic acid targets for each sample were estimated by using the software of the Mx3000P® qPCR system.

2.1 Standardized cell culture infectivity assay (Publication 1, 2 and Manuscript 3)

A standard cell culture infection and inactivation assay for *C. parvum* was established through optimization of HCT-8 cell culture infection in 24 and 6 well cell culture plates. HCT-8 cells were seeded into cell culture microplates and confluent monolayers were infected with a range of oocyst numbers. Infected monolayers without any morphological alteration over the experimental period

were considered suitable for assessment of parasite reproduction. Furthermore, a series of heat inactivated (70°C for 1 h) oocyst suspensions were used to determine a suitable inoculation size at which non-invading parasites were completely removable by washing the monolayer (Manuscript 3). For cell culture infection, oocysts were directly excysted on confluent monolayers with 0.4% sodium taurocholate solution (excystation medium in RPMI-1640) for 3 h at 37°C with 5% CO_2 allowing sporozoites to invade the HCT-8 cells. The monolayer was washed by growing medium (RPMI - 1640 with L-glutamine, 10% newborn calf serum and 1% sodium pyruvate) to remove the non infecting parasite materials. The infection was further maintained in growing medium for the entire incubation period. At the end of the particular incubation period the cells were harvested and subjected to DNA extraction (Publication 1, 2 and Manuscript 3). The sensitivity of the cc-qPCR assay was assessed by using different numbers of *C. parvum* oocysts to infect cell cultures. The sensitivity, linearity and reproducibility of the qPCR were also evaluated by using a dilution series of *C. parvum* infection control DNA or plasmid DNA (cloned fragment of hsp70 gene). The reproducibility and reliability of the methods were observed by repeated experiments.

2.2 *In vitro* drug screening (Publication 1)

The suitability of the cc-qPCR assay for screening of anticryptosporidial drugs was investigated by evaluating reference drugs like monensin (sodium salt, Sigma, Steinheim, Germany) and halofuginone bromide (Intervet, Unterschleissheim, Germany). Miltefosine (hexadecylphosphocholine, Orphanidis, Vienna, Austria), a drug used for treatment of *Leishmania* infection, was also tested against *C. parvum* by cc-qPCR. A range of concentrations were applied to non infected and infected monolayers over the entire incubation periods to determine the toxic effect on host cells and inhibitory effect on parasite multiplication in culture, respectively. The drug to be tested for anticryptosporidial activity was added at the final concentration to 1 ml fresh growing medium that was applied to the respective infected cell culture well. DNA was harvested from cultures before adding drug (3 h p.i., initial level of parasite invasion) and then after the end point of incubation with drugs. Percent relative inhibition of parasite multiplication was estimated as compared to non medicated control cultures.

2.3 *In vitro* effects of curcumin on *C. parvum* (Publication 2)

Antiprotozoal activity of curcumin (diferuloylmethane, Sigma- Aldrich, Steinheim, Germany) has been evaluated by assessment of infectivity, invasiveness and multiplication of *C. parvum* in HCT-8 cell cultures. Curcumin was dissolved in DMSO (dimethyl sulfoxide) and fresh preparations at final concentrations were used in all experiments. Potential toxicity of curcumin to HCT-8 cells was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide)]. The suitability of curcumin pre-treated HCT-8 cells to support *C. parvum* infection was also tested. The infected culture was kept in contact with curcumin over the entire incubation period of 24 h by replacing medium with fresh medicated medium at 12 h intervals. Resume of parasite multiplication was determined following withdrawal of drug exposure for 24 h. To study the effects on infectivity oocysts were pre-treated with curcumin for 12 or 24 h at room temperature before being inoculated into monolayer. The effect of curcumin on invasiveness of sporozoites was evaluated in the presence of curcumin during the excystation and invasion period of 3 h. Curcumin induced effects were demonstrated by comparison with non treated monolayers. Monensin (0.144 µM, Sigma) was used as a reference anticryptosporidial drug.

2.4 *C. parvum* oocyst inactivation studies (Manuscript 3)

The suitability and reproducibility of the cc-qPCR assay for inactivation studies were tested by evaluating DVG approved cresol based anticoccidial disinfectants (Neopredisan® 135-1, Menno Chemie, Norderstedt, Germany; Aldecoc® TGE, EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany) and by using thermally treated oocysts. Other products not approved as anticoccidial disinfectants applied were Aldecoc® XD (EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany), IGAVET® FF spezial (aldehyde, COS OHLSEN Chemie & Gerätevertrieb GmbH, Geltorf-Esprehm, Germany), bleach (sodium hypochlorite) and KokziDes® (Arthur Schopf Hygiene GmbH & Co. KG, Neubeuern, Germany), a disinfectant not yet commercialized. The cc-qPCR was further analysed for its suitability as an alternative to a standardized chicken infection model (DAUGSCHIES et al. 2002) that is approved by DVG for routine screening of anticoccidial disinfectants. For inactivation assays, 1 x 10^6 oocysts were subjected to different treatments over different periods and subsequently washed with tap water. The size of inoculum (5 x 10^4) was accordingly adjusted. Percent inactivation was calculated by comparing the parasite DNA copies in

treated cultures to that of untreated controls. In order to compare inactivation determined by the cc-qPCR model with inactivation determined by the chicken model as previously reported, a log-dose response (log inoculum-log parasite DNA copies) calibration curve was generated from parasite DNA isolated from cell cultures infected with a dilution series of oocysts. The respective data were transformed into a log dose-response calibration curve to back calculate the number of infective oocysts (ID) remaining in each inoculum after treatment by using the formula $y = mx + b$ ('x' is log ID, 'y' is log DNA copies, 'a' is slope and 'b' is intercept). Percent inactivation was calculated as the difference of ID values between treated and untreated control samples. A threshold value was proposed for certification of a product to be sufficiently effective against coccidia.

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Combination of cell culture and quantitative PCR for screening of drugs against *Cryptosporidium parvum*

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ABSTRACT

Cryptosporidium parvum is a zoonotic pathogen causing self-limiting diarrhea in immunocompetent patients. An assay combining cell culture and real time quantitative PCR (qPCR) is reported here to verify drug efficacy against *C. parvum* in vitro. The monolayers of Human ileocecal adenocarcinoma cells (HCT-8) were infected by sporozoites excysted directly on the cells and were incubated with monensin, halofuginone bromide and hexadecylphosphocholine until 45 h post infection (p.i.). The genomic DNA was extracted at 3, 27 and 45 h p.i. and subjected to qPCR targeting the 70 kDa heat shock protein gene to quantify the development of *C. parvum*. The reliability of the method was validated by testing of monensin and halofuginone bromide, which are well known to be effective in vitro. With the dose dependency monensin and halofuginone showed a maximum inhibition of 98.15% and 98.05% at 0.144 and 25 μM, respectively, compared with non-treated controls at the endpoint incubation, confirming previous reports. The reduction of the parasite DNA reproduction over 27 h p.i. compared with 3 h p.i. was found to be as 97–99% in 0.144 μM monensin and 99% in 25 μM halofuginone treated cells. The new antileishmanial compound hexadecylphosphocholine (24.5 μM, Miltefosine®) showed 78–98% inhibition at 45 h p.i., however, the reproduction of parasite DNA was reduced to 96–98% over 27 h p.i. The method has the potential to easily and reliably assess anticryptosporidial compounds in adequately equipped routine laboratories.

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1. Introduction

Cryptosporidium parvum is an apicomplexan parasite and a common cause of diarrhea in many animal species and man. Infection is mostly mild and self-limiting in immunocompetent individuals but may cause severe disease in immunocompromised patients (Chappell and Okhuysen, 2002). Animal models are generally used to determine infectivity of parasite isolates and to test for example efficacy of drug preparations against *C. parvum*. However, animal models are laborious, expensive, time-consuming and related to ethical concern. In vitro infection methods are, at least partially, suited to replace animal infection models (Armson et al., 1999; Shin et al., 2001; Rochelle et al., 2002; Jenkins et al., 2003). Detection and quantification of intracellular stages in cell cultures have been achieved by several methods like conventional light microscopy (McDonald et al., 1990; Armson et al., 1999), focus detection method (Slifko et al., 1997), ELISA (Woods et al., 1995; Gargala et al., 1999), rapid semi-quantitative screening method (Armson et al., 1999) and recently semi-automatic detection of immunofluorescence by computerized image analysis (Najdrowski et al., 2007). Quantitative (q)PCR is considered to be particularly sensitive, specific and reproducible (Godiwala et al., 2006) and is widely applied in *Cryptosporidium* research (Fontaine and Guillot, 2002) including in vitro and in vivo assessment of infectivity (MacDonald et al., 2002; Cai et al., 2005;

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Di Giovanni and LeChevallier, 2005). We report here an in vitro model suited to easily and reliably quantify reproduction of *C. parvum* by combination of cell culture and qPCR to test for example drug effects or efficacy of oocyst inactivation measures.

2. Materials and methods

2.1. Cloning procedures—standard preparation

The qPCR standard was generated by serial dilution of a cloned amplification target (fragment of hsp70 gene). For this purpose the plasmid DNA of the pBluescript® II SK (+) Phagemid (Stratagene, Genbank Acc. No. X52328) was constructed as T-vector using SmaI restriction enzyme as described previously (Marchuk et al., 1991). The PCR products generated by Taq polymerase were used for ligation and transformation of competent cells as described elsewhere (Dyachenko et al., 2008). The plasmid DNA was extracted by QIA prep® Spin Miniprep Kit (Qiagen, Hilden, Germany) and linearized with restriction enzyme (SacI) and purified by DNA clean and concentrator™ (Zymo Research, California, USA). The concentration was measured by spectrophotometry (Eppendorf, Hamburg, Germany) and finally, aliquots of about 3.345 ng plasmid DNA (equivalent to 10^9 copies or molecules) and different standard dilutions were made and preserved at −20 °C.

2.2. Parasite, host cell and compounds

The experimental strain of *C. parvum* (GP-60 subtype IIa A15G2RI) was isolated in Wilchwitz, Germany in the year of 2005 and was maintained by serial passages through infecting a day old calf orally with of 9×10^6 viable oocysts. The calf developed diarrhoea and excreted oocysts from day 4 after infection until day 13. Oocysts were collected, cleaned and isolated according to the procedure described by Najdrowski et al. (2007). The isolated oocysts were stored in phosphate buffered saline (PBS, pH 7.4) with streptomycin (200 μg/ml) and amphotericin B (5 μg/ml) at 4 °C. The storage medium was changed at monthly intervals. Human ileocecal adenocarcinoma cells (HCT-8) were cultured in growing medium (RPMI-1640 with L-glutamate, 10% newborn calf serum, 1% sodium pyruvate) and incubated at 37 °C with 5% CO_2. Monensin (sodium salt, Sigma, Steinheim, Germany) was dissolved in DMSO, halofuginone bromide (Intervet, Unterschleissheim, Germany) in sterile tap water and miltefosine (Orphanidis, Vienna, Austria) in 5% alcohol to make a stock solution. Stock solutions of the chemical compound with exception of miltefosine were stored at −20 °C until use.

2.3. In vitro infectivity inhibition assay

HCT-8 cells were seeded in 24 well (2×10^5/well) and also in 6 well (1×10^6/well) cell culture microplates, allowing them to develop to monolayers in growing medium at 37 °C with 5% CO_2. When the monolayer was nearly confluent, it was washed with growing medium before oocysts (5×10^4/well in 24 well microplates; 2.5×10^5/well in 6 well microplates) were inoculated. Oocysts were pre-treated with 5.25% sodium hypochlorite (12% active chlorine, original stock) and added to HCT-8 monolayers together with sterile 0.4% taurocholic acid sodium salt hydrate (Sigma, Steinheim, Germany) solution (excystation medium) and incubated for 3 h. The non-infecting parasites and oocysts were washed out from the wells by an exchange of excystation medium with fresh growing medium. Thereafter, the drugs to be tested for anticryptosporidial activity were added at the final concentration of monensin (0.0002304, 0.001152, 0.00576, 0.0288, 0.144 μM), halofuginone (0.04, 0.2, 1, 5, 25 μM) and miltefosine (0.0392, 0.196, 0.98, 4.9, 24.5 μM). Infected positive controls and uninfected controls (HCT-8) did not receive any drugs. Infected and uninfected monolayers were further incubated until 15, 27 and 45 h post infection (p.i.) at 37 °C and 5% CO_2. The monolayers were inspected by light microscopy daily. In parallel, a similar number of oocyts were excysted in 1.5 ml tube and percent excystation was calculated according to the formula

$$\frac{\text{excysted oocysts}}{\text{total oocysts}} \times 100.$$

2.4. DNA extraction

After 3, 15, 27 and 45 h p.i. (time count from addition of excystation medium) the monolayers were washed 3 times with sterile PBS containing Ca^{2+} and Mg^{2+}. The monolayers were treated with accutase (PAA Laboratories GmbH, Coelbe, Germany) at 37 °C and the detached cells were transferred to microcentrifuge tubes. After centrifugation ($1540 \times g$ for 5 min) the cell pellets were resuspended in 200 μl PBS and subjected to DNA extraction applying the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (blood and body fluid spin protocol). The DNA was eluted with 50 μl ddH_2O and the concentration was measured by spectrophotometry (Eppendorf, Hamburg, Germany) at 260 nm. DNA was stored at −20 °C until use.

2.5. qPCR assay

qPCR was performed with primers and probe (TaqMan) specific for the *C. parvum* hsp70 gene (forward primer: CP_hsp70_fwd (2219–2246) 5′-aactttagctccagttgagaaagtactc-3′; reverse primer: CP_hsp70_rvs (2336–2362) 5′-catggctctttaccgttaaagaattcc-3′); TaqMan probe: (HSP_70_SNA 5′-aatacgtgtagaaccaccaaccaatacaacatc-3′) designed by Michael Lebuhn (personal communication; October 13, 2008). The PCR reactions were prepared in a total volume of 25 μl consisting of 1× PCR buffer (ABgene mastermix, AB gene limited, Surrey, England) containing dNTP, $MgCl_2$ and Taq-polymerase; 300 nM CP_hsp_70_fwd and 900 nM CP_hsp_70_rvs (each 25 μM, Biopolymer, Ulm, Germany); 30 nM of ROX reference dye; 200 nM of HSP_70_SNA (25 μM, Biopolymer, Ulm, Germany) labelled at the 5′-end with the 6-carboxyfluorescein reporter dye (FAM) and at the 3′-end with the 6-carboxytetramethylrhodamine quencher dye

(TAMRA). The used final concentrations of primers and probe were found empirically in previous optimization procedures. A plasmid standard or target sample template DNA was added to each reaction tube. Negative controls with HCT-8-DNA and no template control were included in each assay. The amount of template DNA used in each reaction ranged from 120 to 700 ng. The qPCR reactions were run in a Mx3000P® QPCR system (Stratagene, La Jolla, USA) with an initial polymerase activation step at 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s and at 60 °C for 1 min.

2.6. Data analysis

The results of real-time PCR assay were analysed by the software incorporated with MX3000P (Stratagene®, La Jolla, USA). Microsoft Excel (Microsoft Corp., WA, USA) on a conventional PC was used for graphs and analysis for correlation (r^2) between concentrations of drug and their effects on multiplication of *C. parvum* in vitro. Non-linear regression using sigmoidal model was used to calculate the EC_{50} values (effective concentration 50%) in the GraphPad Prism® version 5.0 (GraphPad software Inc., La Jolla, CA, USA).

3. Results

3.1. Cell culture infection

The first step in the effort to establish a standardized infectivity or inactivation assay was the optimization of the culture system and of infection of HCT-8 cells in 24 and 6 well cell culture plates. A range of 1.5×10^5 to 3×10^5 HCT-8 cells per well were seeded into 24 well microplates and monolayers were infected with oocysts at doses of 1×10^4 to 5×10^5. For 6 well microplates an initial density of 2×10^5 to 1×10^6 HCT-8 host cells is compatible with an inoculation dose of 1×10^6 to 3×10^6 oocysts. Inoculation of cultures containing low numbers of cells with high numbers of oocysts produced morphological alteration of the monolayer and host cells, respectively. The best combination appeared to be 2×10^5 HCT-8 with 5×10^4 oocysts for 24 well microplates and 1×10^6 HCT-8 with 2.5×10^5 oocysts for 6 well microplates. With these combinations the optimal period for assessment of parasite proliferation (maximum parasite numbers) in the controls was 48 h.

Viability of the oocysts was determined by percent excystation. About 1–9-month-old oocysts were used in these experiments. The rate of excystation (81–60%) and percent infectivity in cell culture varied (84.9–33.3%) with the age of oocysts. It thus appeared that, in addition to age dependent excystation rates, not all excysted sporozoites were infective. Because the excystation rate does not appropriately reflect viability and infectivity, the percent infectivity of oocysts inoculated into cell cultures was calculated according to DNA amplification by the following formula:

$$\frac{\text{number of intracellular parasites in control well at 3 h}}{\text{number of parasites present in single inoculation dose}} \times 100$$

In parallel to cell culture infection, a similar number of oocysts were incubated for excystation in host cell free medium for 3 h at 37 °C with 5% CO_2. Thereafter, DNA was extracted.

3.2. qPCR validation

Sensitivity, reproducibility and linearity of the qPCR reaction were tested by serially diluting DNA prepared from untreated positive control wells infected with the highest number of oocysts (5×10^4). Assay sensitivity and reliability were also tested in five trials by using linearized or non-linearized plasmid standard with or without adjusting the total genomic DNA at a constant level (300 ng). A standard curve was drawn by plotting C_t values (cycle threshold) against the log number of DNA copies obtained from qPCR (Fig. 1). Correlation (r^2) values ranged from 0.994 to 1.000 and correlation was highly significant ($P < 0.001$). Efficiency of qPCR was high with values of 92.9–98.7%. Considering the total length of 3105 bp (insert 144 bp+ pBluescript® II SK (+) 2961 bp) copy numbers of 10^9 were calculated corresponding to the amount of 3.3 ng DNA. The detection limit was 10^2 copies for plasmid DNA or 25 oocysts corresponding to $25 \times 4 = 100$ sporozoites.

The reproducibility of the C_t values applied for calculating the number of DNA copies was assessed using

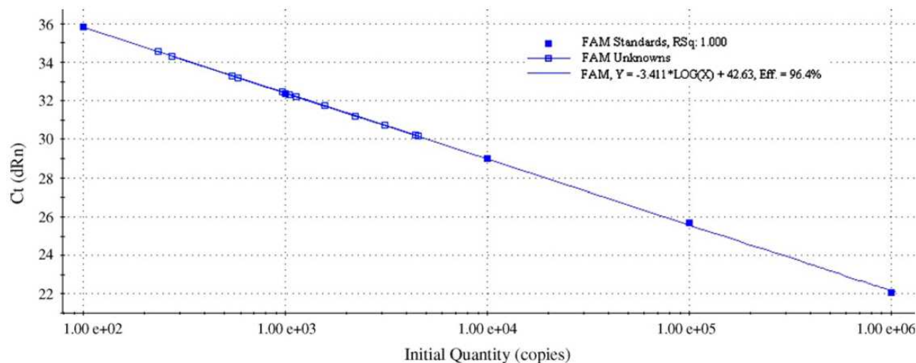

Fig. 1. Standard curve generated from serially diluted plasmid DNA.

Table 1
Percent inhibition of monensin (0.144 μM) and halofuginone (25 μM) at 27 h p.i.

Experiments	Percent inhibition	
	Monensin	Halofuginone
Trial 1	97.26	98.59
Trial 2	98.94	98.55
Trial 3	99.48	97.15
Trail 4	96.9	97.9
Mean	98.15	98.05
SD	1.09	0.59
CV (%)	1.11	0.60

% inhibition = [(DNA copies in control well − DNA copies in treated well)/ DNA copies in control well] × 100. 1.11% and 0.60% CV (coefficient of variation), indicating a high level of reproducibility.

three aliquots of the same DNA samples in each case. The mean standard deviation did not exceed 0.5, indicating high reproducibility (Heid et al., 1996). Further validation was achieved by determining variance of the results of four repeated experiments which demonstrated low variation and thus a high level of reproducibility of the assay (CV = 0.60–1.11%) (Table 1).

3.3. Effects of drug exposure

The toxicity of the inoculated drugs on the cells at the applied dose levels was assessed by microscopical estimation of morphological alterations of the monolayers and individual cells. Irrespective of the applied dose monensin did not induce detectable morphological alterations within the maximum incubation period of 45 h. On the other hand halofuginone induced alterations up to 1 μM only after 45 h and of 5 μM after 15 h. Miltefosine did not induce any detectable toxicity to cells at a dose of up to 4.9 μM until 45 h of incubation whereas some toxicity appeared after 45 h of incubation at a concentration of 24.5 μM (Table 2).

Table 2
Effects of drug on cell culture monolayer and host cell.

Concentration of drugs	Host cell toxicity (hours of incubation)		
	15	27	45
Monensin (μM)			
0.144 μM	–	–	–
0.0288	–	–	–
0.00576	–	–	–
0.001152	–	–	–
0.0002304	–	–	–
Halofuginone (μM)			
25	+	+	++
5	+	+	++
1	–	–	+
0.2	–	–	+
0.04	–	–	–
Miltefosine (μM)			
24.5	–	–	+
4.9	–	–	–
0.98	–	–	–
0.196	–	–	–
0.0392	–	–	–

Symbol (+) indicates degree of alteration (sloughing off cells).

Table 3
Effect of drug exposure on relative multiplication of copy numbers (reference value: 3 h p.i.).

Parameters	Relative copy numbers/(% relative inhibition[a])			
	3 h	15 h	27 h	45 h
Monensin-0.144 μM	10	29.97/(85)	7.2/(97)	11.7/(99)
Halofuginone-25 μM	10	65.6/(68)	3.7/(99)	–
Miltefosine-24.5 μM	10	50.8/(75)	10.7/(96)	18.5/(98)
Control	10	204.3	264.1	1077.9

[a] % relative inhibition = 100 − percent relative multiplication of copy number compared to control.

3.4. Characterization of drug efficacy

Parasite copy numbers were quantified by qPCR at 3, 15, 27 and 45 h p.i. Considering 3 h p.i. as starting point, we calculated the fold changes in parasite stages by dividing the number of copies found at 15, 27 and 45 h p.i. by that of the initial copy number at 3 h p.i. (Table 3; Fig. 2). Altogether qPCR measurements of parasitic load in control wells reflect the success of infection as well as multiplication over the experimental period.

Multiplication at 15 h p.i. was approximately 30-fold for monensin, 66-fold for halofuginone and 51-fold for miltefosine and 204-fold in the non treated positive controls, compared to the initial reference value (3 h p.i.). This corresponds to 85% (monensin), 68% (halofuginone) and 75% (miltefosine) reduction in multiplication, respectively.

Inhibition after 27 and 45 h p.i. was found to be much more pronounced than after 15 h p.i. After 27 h p.i. copy numbers of the controls were 264-fold increased but distinctly reduced in the treated cultures, resulting in relative reduction of copy numbers of 97% (monensin), 99% (halofuginone) and 96% (miltefosine) (Table 3).

Although copy numbers slightly increased in the treated cultures until 45 h p.i., the relative multiplication was further dramatically inhibited compared to the positive controls were the values were 1078 times higher compared to the initial copy number, indicating further multiplication of the parasite. Calculation of the respective relative reduction in copy multiplication gave values of 99% and 98% for monensin and miltefosine, respectively. Due to insufficient number of cells in halofuginone treated

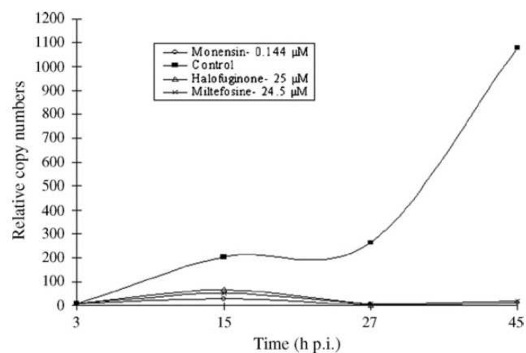

Fig. 2. Effect of drug exposure on relative multiplication of copy numbers (reference value: 3 h p.i.).

wells at 45 h p.i., data for this compound were not available (Table 3).

Thus the applied drugs resulted in a dose dependent reduction of parasite development when compared to the controls in the assays. A significant ($P < 0.05$) dose dependent inhibition (0.001–98%) was found in monensin (0.0002304–0.144 μM) treated cultures with EC_{50} value of 0.002 μM. The concentrations and percent inhibition for halofuginone (0.04–25 μM; 74–99%) and miltefosine (0.0392–24.5 μM; 14–81%) were correlated at a dose dependent manner ($P = 0.05$) (Fig. 3a–c) with EC_{50} values of 0.15 and 1.87 μM, respectively.

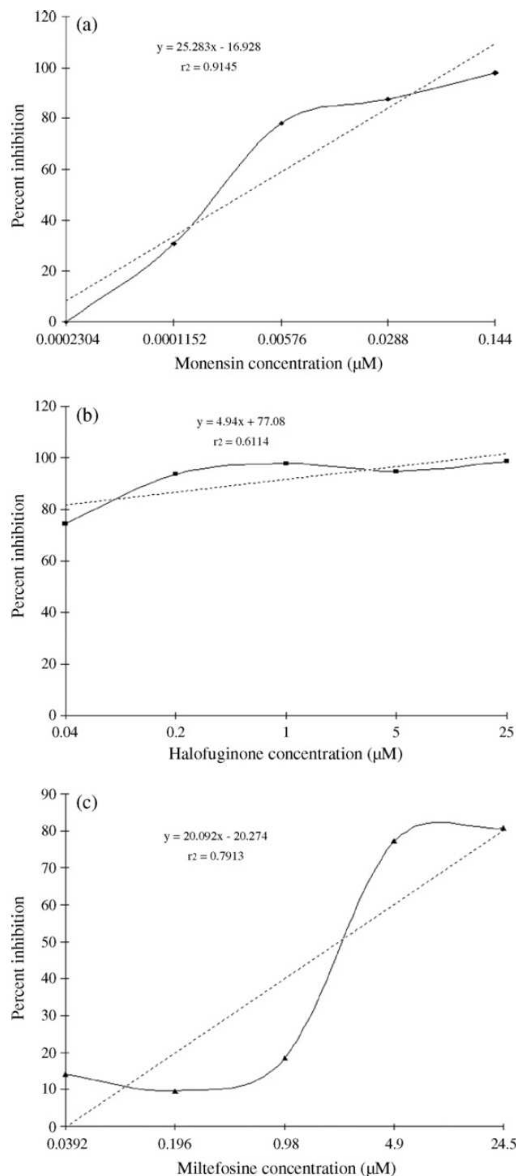

Fig. 3. (a) Dose dependent inhibition on the multiplication of *C. parvum* in vitro at 27 h p.i. (b) Dose dependent inhibition on the multiplication of *C. parvum* in vitro at 27 h p.i. (c) Dose dependent inhibition on the multiplication of *C. parvum* in vitro at 24 h p.i.

4. Discussion

Considering the major impact of cryptosporidia on animal and human health and the lack of specific drugs, reliable rapid methods for testing potential anticryptosporidial drugs or inactivation measures are most valuable. Animal infection models are the 'gold standard' of efficacy testing, however, they have certain limitations with respect to drug screening (costs, labour, premises, animal welfare considerations, etc.). In vitro methods do not suffer from such limitations, however, they are generally not suited to entirely replace drug efficacy testing in animals. Nonetheless, advancements in in vitro screening may speed up and improve the search for, e.g. anticryptosporidial pharmaceuticals.

Cell cultures are suited to assess the infectivity and multiplication of cryptosporidia under controlled and standardized conditions. A number of different cell lines have been successfully applied in the past for in vitro studies on *C. parvum* and HCT-8 cells are believed to represent an ideal cell line (Arrowood, 2002). In our hands, this cell line appeared to be very reliable in terms of monolayer growth and invasion by *C. parvum*.

Several methods such as conventional light microscopy, focus detection method, computer assisted image analysis, etc., have been suggested and applied for detection and quantification of parasite proliferation within cell cultures (McDonald et al., 1990; Slifko et al., 1997; Armson et al., 1999; Najdrowski et al., 2007). Several approaches have been reported to quantify parasite development by qPCR techniques (Fontaine and Guillot, 2002; MacDonald et al., 2002; Di Giovanni and LeChevallier, 2005; Cai et al., 2005). In our study, qPCR based on TaqMan fluorogenic detection of DNA amplified from the specific target sequence of the heat shock protein (hsp) 70 gene was used for assessment of in vitro multiplication of *C. parvum*.

Improvement of cell culture infection through optimised combination of HCT-8 cell seed and inoculation dose of oocysts provided us a confluent (infected) monolayer over the entire observation period which is a prerequisite for accurate and reproducible estimation of parasite multiplication.

Calculation of percent inhibition in wells containing drugs in certain concentrations by simply comparing multiplication with that in simultaneous control wells at a given time does not always allow accurate estimation of inhibition. This is due to the many possible variations, e.g. rate of excystation or variable infectivity of the inoculum that may lead to diverse results in different wells. To avoid these unwanted interferences, we propose to use the actual level of parasite invasion before drug application as a basic value for assessment of parasite proliferation over a given period thereafter. In our hands a period of 3 h after parasite inoculation appeared suitable as a "starting point" and thus the initial copy number of parasite DNA was estimated at this time in each trail.

If the copy number measured in assay wells is distinctly lower than in the control wells this should not be

uncritically addressed to drug induced inhibition. Degradation of DNA in infected dead cells or sloughing of host cells have to be considered. If the copy number is reduced due to drug application this may be related to either parasitocidal effects or host cell toxicity, and therefore toxic effects of the drug under investigation should be assessed in non-infected monolayers before inhibition assays are performed. If the copy numbers remain unchanged within the given period of incubation this indicates a parasitostatic effect of the drug. To draw justified conclusions on efficacy and mode of action (parasitostatic or parasitocidal) of a drug in an assay like this accuracy of performance and knowledge on the characteristics of the drug (e.g. cell toxicity) are essential.

Inevitable variations in the rate of excystation and quality of the inoculum (oocysts) do not considerably affect the results of the proposed in vitro testing system provided that the respective controls (positive, negative, "starting point") are included to allow proper calculation of percent inhibition for each assay (Table 3).

The calculation of a standard curve is crucial to validate the quality of each single assay and to deduce accurate results. One obvious means is serial dilution of inocula of *C. parvum* oocysts, however, due to inevitable variability in oocyst numbers applied to the different monolayers (e.g. because of uneven distribution of oocysts in suspensions or dilution effects), and the inherent biological variability in infectivity, multiplication and type of intracellular developmental stages, the copy numbers of the genomic target will also vary. This may alter the accuracy of standard curves based on titration of oocysts to cell cultures, and as r^2 decreases it will be more difficult to determine the exact location of the standard curve plot and to obtain correct quantification.

Data generated from a serial dilution of a positive control DNA template is an excellent alternative means of determining the overall performance of qPCR assay ($r^2 = 0.9931$). In addition, we have tested the variability in linearity obtained from plasmid standards in our assay and results suggest that quantification of parasitic load based on a respective standard curve is independent on whether linear or non-linear plasmid DNA is used and on the size of the microplate wells. The use of an equal amount of genomic DNA in each reaction and of a wide range of template DNA (120–700 ng) did not significantly alter the procedure, indicating flexibility and simplicity of this method.

The time of assessment after inoculation of cell cultures may have a significant impact on the results. The longer the incubation time the higher the probability of detecting successful infection. However, qPCR is highly sensitive with a detection limit as low as 100 parasite stages (sporozoites), and therefore positive results can be recorded early after inoculation. Thus the selection of the appropriate time for quantification of parasite stages by qPCR in a drug assay depends more on the time required for maximum inhibition by the drug to be tested than on the dynamics of parasite multiplication.

To evaluate the suitability and to further validate the developed assay the inhibitory effects of anticoccidial compounds, monensin sodium salt and halofuginone bromide, on *C. parvum* were determined. Monensin has been well documented as an effective drug against *C. parvum* in cell cultures (McDonald et al., 1990; Armson et al., 1999). At the concentration of 0.0002304–0.144 μM the inhibitory efficacy of monensin (Fig. 3a) increased in a dose dependent manner from 0% to 98% without exerting any significant alterations to the cell monolayer. These observations are consistent with data reported earlier (McDonald et al., 1990; Armson et al., 1999; Najdrowski et al., 2007). Halofuginone is approved for clinical treatment of cryptosporidiosis in calves, however, with limited efficacy (Villacorta et al., 1991; Lefay et al., 2001; Joachim et al., 2003). Under in vitro conditions halofuginone was more than 90% effective (Fig. 3b) but it showed some toxicity to the host cell after 15 h of incubation at a concentration range of 1–25 μM (Table 1). At a concentration of 1 μM almost 98% efficacy was seen, paralleled by some toxicity after 45 h of incubation. This is in accordance with previous results (Najdrowski et al., 2007).

We also employed cell culture combined with qPCR to evaluate the efficacy of miltefosine on the growth of *C. parvum* in vitro. Miltefosine (hexadecylphosphocholine) was originally developed as an antitumor agent and is currently being used as a drug against *Leishmania* and some other protozoa and fungi (Saraiva et al., 2002; Blaha et al., 2006; Widmer et al., 2006; Choubey et al., 2007). To our knowledge this molecule has not been previously assessed for anticoccidial or anticryptosporidial efficacy. In fact, at concentrations above 4.9 μM miltefosine displayed inhibition on the growth of *C. parvum*, whereas inhibition was below 10% and thus insignificant at lower concentrations. Clear cut dose dependency was not seen at the concentration steps tested (Fig. 3c). Death of some host cell was recorded after 45 h at the highest dose, which may be due to strong suppressive effects of miltefosine on the biosynthesis of phosphatidylcholine in mammalian cells (Haase et al., 1991). These observations suggest that miltefosine may be explored as a potential drug for the control of *C. parvum* infection. However, to establish whether or not miltefosine is actually suitable as an anticryptosporidial compound will require testing in animal models reinforcing the general limitations of in vitro testing.

In conclusion, the combination of in vitro culture and qPCR is a suitable method to assess infectivity of *C. parvum* oocysts and to screen potentially anticryptosporidial compounds as confirmed by demonstrating efficacy of monensin and halofuginone. Miltefosine displayed inhibitory activity under in vitro conditions, albeit on a lower level than monensin and halofuginone. The method is simple, rapid, sensitive, reproducible and flexible and may be easily established in suitably equipped laboratories.

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ORIGINAL PAPER

Effects of curcumin on *Cryptosporidium parvum* in vitro

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Abstract *Cryptosporidium parvum* is a zoonotic protozoan parasite having peculiarities among the apicomplexa that could be responsible for its resistance to some drugs and disinfectants against coccidia. The awareness of *Cryptosporidium* as a health problem in man and animal is increasing and potent drugs are urgently needed. Curcumin, a natural polyphenolic compound, has been found to be active against a variety of diseases including anticarcinogenic, antimicrobial, and antiprotozoal effects. We investigated the effects of curcumin on infectivity and development of *C. parvum* in a recently established in vitro system combining infection of human ileocecal adenocarcinoma cell cultures with quantification of intracellular parasites by quantitative polymerase chain reaction. Curcumin was found to be effective (>95% inhibition of parasite growth) at 50 µM for 24 h when infected cultures were exposed for more than 12 h. Withdrawal of curcumin after 24 h of exposure did not result in a significant resumption of *C. parvum* growth. The invasion of host cells by sporozoites (infectivity) was found to be inhibited at least 65% in the presence of 200 µM curcumin. No significant reduction of viability of *C. parvum* oocysts after incubation with curcumin was recorded. Altogether, curcumin showed promising anticryptosporidial effects under in vitro conditions and deserves further exploration.

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Introduction

Cryptosporidium parvum is a zoonotic parasite that causes significant and durable health problems in immunocompromised individuals while self-limiting disease is generally observed in immunocompetent hosts. It is considered as a potent food and drinking water contaminant (Casemore et al. 1997; Blackburn et al. 2004). With increasing awareness of *Cryptosporidium*, effort is continuing to develop new potent drugs and respective experimental tools. Several studies have been performed, however, with limited success regarding efficient control measures. Scientific interest on natural products with anticryptosporidial properties is increasing in recent years. Curcumin is a yellow natural polyphenolic compound extracted from turmeric root (*Curcuma longa*). The active compound (coloring agent) diferuloylmethane [1,7-bis-(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione] is responsible for various pharmacological effects of curcumin. These include antiinflammatory (Ammon and Wahl 1991), apoptotic (Bhaumik et al. 1999; Liu et al. 2005), and antiproliferative (Mehta et al. 1997) activities. The antimutagenic effects of curcumin depend on its antiinflammatory (Rao et al. 1995; Huang et al. 1997) and antioxidant properties (Subramanian et al. 1994). Antiprotozoal activitites of curcumin have been described for *Plasmodium falciparum* (Cui et al. 2007), *Leishmania* spp. (Koide et al. 2002; Das et al. 2008), *Trypanosoma* spp. (Nose et al. 1998), and *Giardia lamblia* trophozoites (Perez-Arriaga et al. 2006). Curcumin-induced effects result from its physicochemical properties. It is relatively stable at low pH, but rapidly decomposes at pH above neutral (Tonnesen and Karlsen 1985); under basic conditions, protons are discharged from the phenolic group, destroying the structure with incubation time. Curcumin is unstable in buffer solutions (Sasaki et al. 1998), but

relatively stable (50% for 8 h) in cell culture medium containing 10% fetal calf serum and in the presence of light (Wang et al. 1997). Curcumin is more stable in higher serum concentrations (Blasius et al. 2004; Duvoix et al. 2005) in the absence of light. Nevertheless, it is somewhat difficult to maintain a constant concentration of curcumin in cell culture experiments due to its low solubility in aqueous solution and instability during sample preparation. In addition, curcumin degradation products, especially vanillin, are reported as antimutagens (Ohta 1993) and also as powerful scavengers depending on the concentration and time of incubation (Liu and Mori 1993). Considering these limitations, precautions must be taken to keep curcumin stable during cell culture experiments to observe the activity of curcumin rather than its degradation products. In vitro drug testing is an early step of drug development and evaluation. We have recently developed a rapid, suitable, and reliable cell culture model for screening drugs against *C. parvum* (Shahiduzzaman et al. 2009). Our present study was performed to evaluate the effects of curcumin on reproduction and infectivity of *C. parvum* in cell culture as quantified by real-time polymerase chain reaction (PCR).

Materials and methods

Cell culture infection

C. parvum oocysts were obtained from an experimentally infected day-old calf. Oocysts were isolated according to the protocol described by Najdrowski et al. (2007). Human ileocecal adenocarcinoma (HCT-8) cells were used for in vitro culture of *C. parvum*. The infection was performed as described by Shahiduzzaman et al. (2009). In short, 2×10^5 HCT-8 cells were seeded into 24-well microplates in growing medium (RPMI-1640 with L-glutamate, 10% newborn calf serum, 1% sodium pyruvate) and incubated to 90% confluency. About 5×10^4 oocysts were directly excysted on the confluent HCT-8 cell monolayer in excystation medium (0.4% sodium taurocholic acid sodium salt) and incubated for 3 h at 37°C with 5% CO_2. Subsequently, unbound parasites were removed by washing with growing medium and the infected monolayers were further maintained in fresh growing medium. Cell culture infection by a dilution series of oocysts (1×10^6 to 10) was used to calculate a standard curve for estimation of infectivity.

HCT-8 cells viability

Curcumin (Sigma-Aldrich, Steinheim, Germany) was dissolved in dimethyl sulfoxide (DMSO) as 100 mM (36.84 mg/ml) stock solution and filtered through sterile filters (0.2 µm, Renner GmbH, Dannstadt, Germany). Working concentrations (3.125 to 200 µM) were prepared in fresh growing medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the threshold of toxicity of curcumin for HCT-8 cell culture. MTT was dissolved in RPMI-1640 growing medium as 5 mg/ml stock solution and diluted 1:10 to make a working solution. HCT-8 cells (3×10^4/well) were seeded in 96-well microplates and incubated to 90% confluence at 37°C with 5% CO_2. Curcumin was added to the wells and the cultures were further incubated. After 3, 12, and 24 h post-initiation exposure (freshly prepared medicated medium was added at 12 h interval), the cells were washed, and 100 µl freshly prepared working solution of MTT was added for a further incubation period of 3 h. MTT solution was removed, and 100 µl acidic isopropanol (0.04 M HCl in absolute isopropanol) was added in both treated and non-treated wells to dissolve the converted dye (formazan). Supernatant was collected in 1.5 ml tubes and centrifuged at high speed, and optical density was measured spectophotometrically at 595 nm (BioPhotometer, Eppendorf, Hamburg, Germany). Cells without curcumin served as positive control and cells without MTT as negative control. Percent viability was calculated relative to the non-treated control.

In vitro growth inhibition test

C. parvum growth inhibition test was performed with working concentrations of curcumin (3.125 to 50 µM) that was immediately prepared from freshly dissolved curcumin in DMSO. After 3 h of excystation (i.e., immediately after initiation of drug exposure), the remaining sporozoites as well as not excysted oocysts and oocyst walls were washed out, and curcumin was added to the wells. The medicated medium was replaced at 12 h post-initiation of exposure with a fresh preparation of the same concentration of curcumin. After 24 h, curcumin was removed by washing the cells with growing medium, and the cultures were maintained in fresh growing medium for another 24 h. At the end of the incubation period, the monolayers were washed twice with phosphate-buffered saline (PBS), cells were trypsinized with accutase (PAA Laboratories GmbH, Coelbe, Germany), and each specimen was collected in a 1.5-ml tube for subsequent DNA extraction. The following control cultures were used: monensin (0.144 µM, Sigma) treated, infected; infected without drug; DMSO (corresponding to the highest concentration of curcumin), infected. We avoided light exposure whenever possible while working with curcumin.

Since antiproliferative and cytotoxic effects of curcumin on cancer cells are known (Kuttan et al. 2007), uninfected

HCT-8 cells were treated with curcumin, and these cells were tested for suitability to support *C. parvum* growth after subsequent infection. For this purpose, about 80% confluent monolayers were incubated with 25 or 50 µM curcumin for 24 h, and after removing curcumin, the cells were infected as described above and maintained in fresh growing medium for 48 h.

Invasion inhibition assay

For the invasion inhibition experiments, about 5×10^4 oocysts were incubated on cell culture monolayers in excystation medium in the presence or absence of curcumin (25 to 200 µM) for 3 h in 5% CO_2 at 37°C. Curcumin was removed by washing twice the monolayers with growing medium, and cell cultures were maintained for 48 h in growing medium.

Oocyst viability assay

To measure viability of oocysts exposed to curcumin, about 1×10^6 oocysts of *C. parvum* were incubated at room temperature in a 1.5-ml tube in the presence of curcumin (25 to 200 µM) for 12 and 24 h. After washing three times with tap water, the size of the inoculum for infection of cell cultures was adjusted to 5×10^4 oocysts/well in counting chambers. In parallel, thermally inactivated (80°C for 10 min) oocysts were used to estimate efficacy of washing steps. The treated oocysts were inoculated into confluent cell cultures, and growth of *C. parvum* was assessed after 48 h.

For both the oocyst viability and invasion inhibition assays, further washing of monolayers, collection of cells, and extraction of DNA were performed as described above. The percent oocyst viability and invasion inhibition were measured in comparison to the non-treated controls.

DNA processing and quantitative PCR

All DNA samples were prepared according to the guidelines (blood and body fluid spin protocol) for QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) followed by elution of extracted DNA in 100 µl elution buffer supplied with the kit. Extracted DNA was stored at –20°C if not used immediately. The quantitative PCR (qPCR) was performed with the primers (targeting specific sequence of the 70-kDa heat shock protein gene) and probe described in a previous report (Shahiduzzaman et al. 2009). Each 25-µl reaction mixture contained 1× PCR buffer (ABgene mastermix, AB Gene Limited, Surrey, England, UK) containing deoxyribonucleotide triphosphate, $MgCl_2$, Taq polymerase, 300 nM forward primer (*cphsp70*), 900 nM reverse primer (*cphsp70*; each 25 µM, Biopolymer, Ulm, Germany), 30 nM of ROX reference dye (1 mM AB gene), 200 nM of TaqMan Probe (*hsp70 sna*; 25 µM, Biopolymer, Ulm, Germany), and 3 µl template. The reaction was performed in a Mx3000P® qPCR system (Stratagene®, La Jolla, USA) with the condition of enzyme activation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 15 s, and finally annealing and elongation at 60°C for 1 min. Each reaction was run in triplicate. A cell culture standard curve was used to calculate the parasite copy numbers in each experiment.

Data analysis

All data were analyzed using the GraphPad Prism® version 5.0 (GraphPad software Inc., La Jolla, CA, USA). *t* test was performed to compare two samples. Non-linear regression using a sigmoidal model was used to calculate the 50% inhibitory concentration (IC_{50}). Data were formatted to bar graphs using MS Excel (Microsoft Corp., WA, USA).

Results

HCT-8 cells viability

MTT revealed selective toxicity of curcumin to HCT-8 cells that increased with concentration and time of exposure. Curcumin was not toxic to HCT-8 cells at any concentration over 3 h. No significant increase of cell death was found at exposure to 100 µM for 12 h, whereas incubation with more than 50 µM for 24 h induced significant toxicity (Fig. 1). Curcumin was significantly toxic after 48 h even at a lower concentration of 12.5 µM (data not shown). Therefore, we selected a test concentration of curcumin up to 50 µM for 24 h at which not more than 5% reduction of viable HCT-8 cells was observed.

In vitro growth inhibition test

C. parvum growth in cell culture was quantified by qPCR using a standard curve (correlation, $r^2>0.995$) derived from cell culture infection with tenfold serial dilution of oocysts (from 10^6 to 10). The ability of curcumin pretreated HCT-8 cells to support subsequent infection and growth of *C. parvum* was found to be 97% (50 µM; 24 h) and 98% (25 µM; 24 h), and infection levels further increased over time resulting in a growth curve almost identical to that of non-pretreated infection control.

After application of 50 µM curcumin to infected monolayers, the infection level was reduced to 36% after 24 h. The growth inhibitory effects of curcumin were significantly ($p<0.001$) increased when cultures were washed, and freshly prepared medicated medium was applied after 12 h of initial exposure to curcumin, in

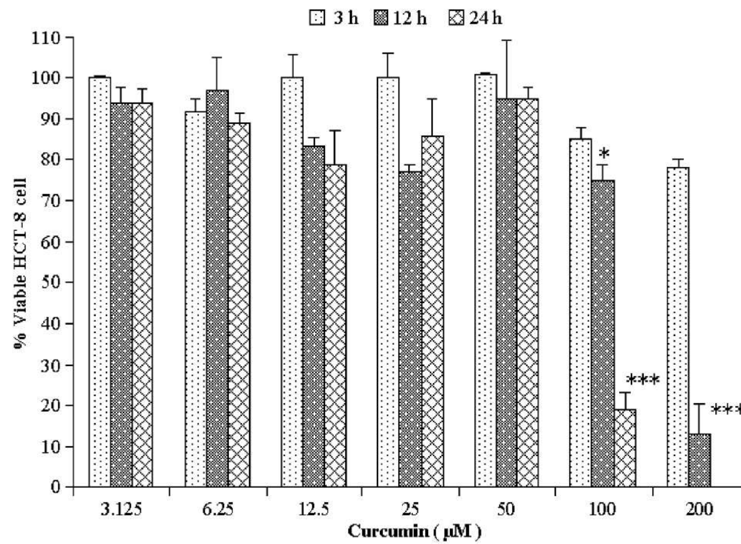

Fig. 1 Viability of HCT-8 cells in curcumin-treated monolayers (control =100%) at 3, 12, and 24 h of exposure. *Asterisks* indicate significant difference (***$p<0.001$; *$p<0.05$, by *t* test) to control. *Error bar* indicates standard deviation of means from three repeated experiments (three sample replicates in each experiment)

comparison to continuously exposed cultures. This was observed for 12.5, 25, and 50 µM curcumin (Fig. 2). Dose-dependent effects were demonstrated by non-linear regression ($r^2=0.942$; Fig. 3) with an IC_{50} value of 13 µM curcumin. Curcumin showed 97% growth inhibition on *C. parvum* in the presence of 50 µM for 24 h. Under these conditions, neglectable toxicity (5% cell death) of HCT-8 cells was seen (no HCT-8 cell death in controls). About 79% growth inhibition was observed at a concentration of 25 µM without any obvious toxicity to HCT-8 cells.

Infected cultures treated for 24 h and then maintained in growing medium without curcumin for another 24 h displayed a significant increase in parasite numbers following withdrawal of curcumin except for those exposed to 50 µM and in some cases to 25 µM curcumin. Monensin (0.144 µM), as a reference drug, allowed no significant ($p>0.05$) resumption of *C. parvum* growth (Fig. 4).

Invasion inhibition assay

The percent invaded/adhered sporozoites as compared to the total number of sporozoites in inoculum (corresponding to the parasite DNA copies obtained from 5×10^4 oocysts/inoculum) was 82% as quantified by real-time PCR. The invasiveness of sporozoites to HCT-8 cells was evaluated in the presence of 25, 50, 100, and 200 µM curcumin. Curcumin exposure during the period of excystation resulted in a dose-dependent decrease in the number of intracellular/adhered sporozoites compared to the control

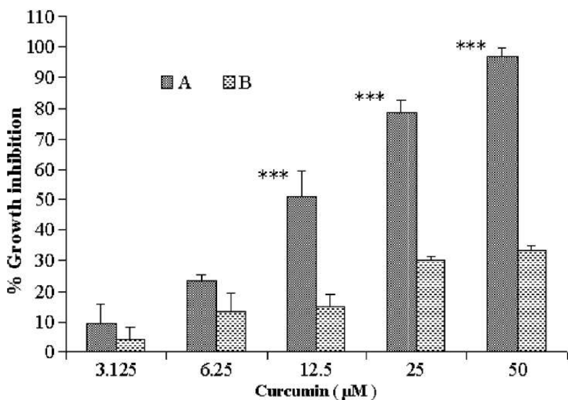

Fig. 2 Efficacy of curcumin on *C. parvum* growth. *Asterisks* indicate significant differences (*t* test, $p<0.001$). *Error bar* indicates standard deviation of three replicates from three repeated trial. *A* Curcumin was replaced by a fresh preparation of medicated medium after 12 h of exposure (24 h of drug contact); *B* no replacement with fresh preparation (24 h of drug contact)

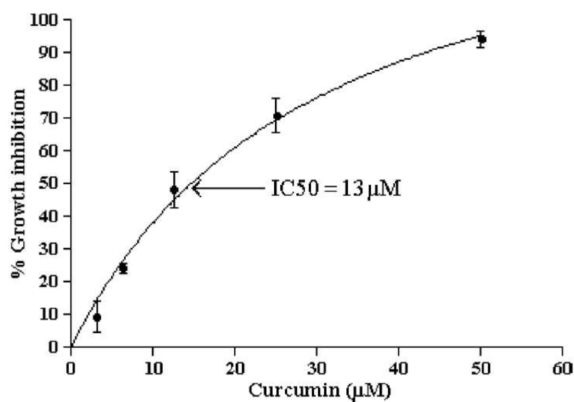

Fig. 3 *C. parvum* growth inhibition curve derived from non-linear regression by using hyperbolic equation in GraphPad Prism® software. *Bar* represents standard error of means from three repeated trials)

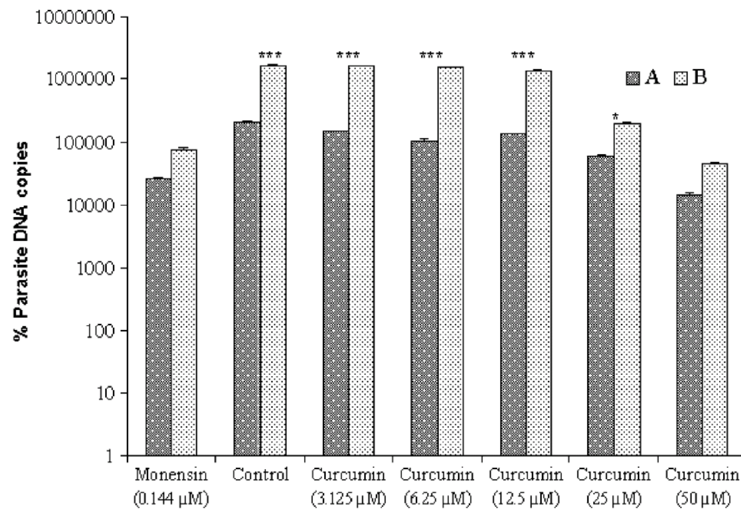

Fig. 4 Resumption of *C. parvum* growth following 24 h of curcumin treatment. *Bar* represents standard error of the mean from three replicates of each sample qPCR. *Asterisks* represent significant difference (*** $p<0.001$; * $p<0.05$, by *t* test) between *A* and *B*. *A* Drug exposure over 24 h after infection (control without drug); *B* drug exposure over 24 h after infection and subsequent incubation over 24 h without drug

(Fig. 5). Curcumin was found to be 65% effective against invasion/adherence of sporozoites to HCT-8 cells with an IC_{50} value of 97.7 µM. No significant toxicity to HCT-8 cells was demonstrated under these conditions ($p>0.05$).

Oocyst viability assay

Application of 25 to 200 µM curcumin displayed no residual effects after washing the treated oocysts with PBS before incubation with HCT-8 cells. The heat-inactivated oocysts that passed through the cell culture did not have any influence (indicating no remaining inert or unexcysted oocysts on monolayer after washing) on detection indicating reliability of the detection assay. In this study, no significant ($p>0.05$) difference in viability of oocysts exposed to curcumin compared to untreated controls was recorded irrespective of the duration of exposure of oocysts, except 200 µM for 12 h (Fig. 6).

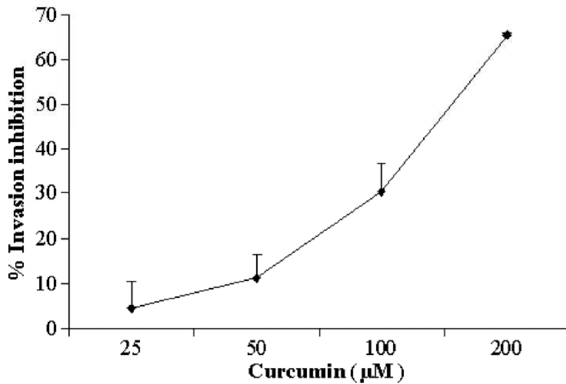

Fig. 5 Dose-dependent inhibition of sporozoite invasion/adhesion to HCT-8 cells. *Error bars* represent standard deviation of means from three repeated experiments

Phase contrast microscopy did not reveal any alteration in the texture of both sporozoites and oocysts after contact with curcumin.

Discussion

Until now, over 200 chemotherapeutic agents have been evaluated against *Cryptosporidium*; some of them are active against other apicomplexa, but none of them is able to clear the parasite from the host. *Cryptosporidium* is notoriously diverged from other apicomplexan parasites by some peculiarities in biology, distinct structure, and biochemical composition that could be related to natural drug resistance. Curcumin, a polyphenolic compound, is increasingly acknowledged as having antimicrobial and antiparasitic activities (Sharma 1976; Araujo and Leon 2001). In vivo effects of curcumin in animal models are well documented for *Leishmania* (Araujo et al. 1998) and *Plasmodium* (Reddy et al. 2005).

Unstability of curcumin in working solution, degradation during sample preparation, or overexposure time (Tonnesen

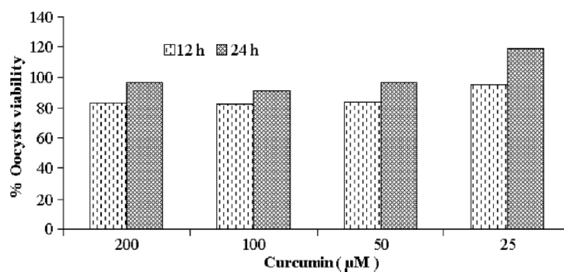

Fig. 6 Range of viability of oocysts exposed to curcumin as compared to control (100%)

and Karlsen 1985) and by light (Sasaki et al. 1998) were taken into account to take full advantage of curcumin against *C. parvum*. Preparation of working solution of curcumin with culture medium containing 10% fetal calf serum protects curcumin from degradation as reported previously (Blasius et al. 2004). Application of fresh curcumin preparation every 12 h is a novel approach that appeared to greatly improve efficacy of the drug against *C. parvum* in vitro development. Selection of the concentrations used and the exposure time were according to previous studies (Wang et al. 1997; Blasius et al. 2004). In our study, curcumin was significantly toxic to HCT-8 cells at exposure over 24 h at a concentration of more than 50 µM. This might be due to a profound increase in the production of reactive oxygen species (ROS) induced by drug exposure (Cao et al. 2006) or less possibly associated with degradation products as reported by Chauret and Boardman (1998). Lower concentrations of curcumin had no considerable negative effects on HCT-8 cells. HCT-8 cells pretreated with curcumin were found to be suitable to harbor *C. parvum*, and thus, no residual effect of the drug was seen.

Curcumin was found to be effective against *C. parvum* in cell culture. The effectiveness was increased when curcumin was replaced by a fresh preparation of medicated medium after 12 h of exposure, and growth inhibition was dose-dependent. At 24 h after inoculation of the cultures, curcumin at 50 µM showed more than 95% efficacy with an IC_{50} value of 13 µM. Curcumin also displayed a potent antiparasitic effect with IC_{50} of 20 to 30 µM on *P. falciparum* in cell culture (Cui et al. 2007). Perez-Arriaga et al. (2006) reported similar growth inhibition of *G. lamblia* trophozoites in vitro in the presence of 30 µM of curcumin over 72 h and of 100 µM over 48 h. An IC_{50} value of 37.6 µM against *Leishmania* in cell culture was reported by Koide et al. (2002). The IC_{50} value of 13 µM in this study indicated that *C. parvum* development appears to be more sensitive to curcumin than that of *Plasmodium*, *Giardia*, and *Leishmania*.

Antiprotozoal activities of curcumin have been attributed to inhibition of histone deacetylation in *P. falciparum* (Cui et al. 2007). Histone deacetylase regulates transcription and is one of the novel therapeutic targets for fungal-derived antiprotozoal agents (like acipidin), thereby such drugs may alter proliferation of apicomplexan parasites such as *C. parvum* (Darkin-Rattray et al. 1996). A new member of the apicomlexan histone deacetylase family has been recently described in *C. parvum* (Rider and Zhu 2009), and it seems to be possible that a respective mechanism is involved in inhibition of growth of *Cryptosporidium* by curcumin. Curcumin at lower doses of 1 to 15 µM scavenges ROS or slightly increases ROS level, but at higher doses (20 to 50 µM) induces oxidative stress (especially H_2O_2) in cancer cells (Cao et al. 2006) and malarial parasites (Cui et al. 2007). Increased production of ROS induced apoptosis in *P. falciparum* (Cui et al. 2007)and DNA damage in *Giardia* (Perez-Arriaga et al. 2006) and leads to death of *Leishmania* (Das et al. 2008). Leitch and Qing (1999) reported that both reactive nitrogen and ROS play protective roles in experimental cryptosporidiosis in mice. *Cryptosporidium* has a poor capacity to scavenge ROS (Entrala et al. 1997), making it potentially more susceptible to killing by such compounds.

Following discontinuation of treatment after 24 h of initial exposure with 25 and 50 µM curcumin, no significant resumption of *C. parvum* growth was observed. In vitro evaluation of monensin (0.144 µM) was in conformity with the results reported by Najdrowski et al. (2007), indicating the reliability of the present curcumin efficacy data in this study. About 65% inhibition of invasion and/or adherence to HCT-8 cells in the presence of 200 µM curcumin was recorded. Curcumin was found to significantly increase adhesion but remarkably reduce viability of *Giardia* trophozoites (Perez-Arriaga et al. 2006). Inhibition of phospholipases (PLA2) and thus of arachidonic acid production was reported to reduce infectivity in *Toxoplasma* (Saffer et al. 1989; Saffer and Schwartzman 1991) and *Cryptosporidium* (Pollok et al. 2003). Curcumin is reported to inhibit mammalian cellular phospholipase (Huang et al. 1991; Rao et al. 1995), and thus, it may be speculated that the reduced infectivity of sporozoites was possibly explored by curcumin.

In conclusion, the sensitivity of *Cryptosporidium* to curcumin in cell culture indicates the presence of respective target molecules in developmental stages. The suitability of curcumin for treating cryptosporidiosis should be further elaborated including animal models and characterization of the putative target sites.

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Combination of cell culture and quantitative PCR (cc-qPCR) to assess disinfectants efficacy on *Cryptosporidium* oocysts under standardized conditions

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Abstract

Oocysts of *Cryptosporidium parvum* are resistant to environmental conditions and many disinfectants. A combination of cell culture and quantitative real time PCR (cc-qPCR) is established for evaluation of anticoccidial disinfectants against *C. parvum*. *Cryptosporidium parvum* oocysts were treated with disinfectants, washed and oocysts were incubated with HCT-8 cell monolayers in presence of excystation medium for 3 h. Subsequently, unbound parasites were removed by washing with growing medium and the infected monolayers were further maintained in fresh growing medium for 48 h. Genomic DNA was extracted from each sample and qPCR performed targeting a specific sequence of the 70 kDa heat shock protein gene in order to quantify development. Treatment of oocysts with cresolic disinfectants demonstrated dose dependent reduction of viability of oocysts. More than 98% inactivations were recorded with at least 2% concentration of cresolic disinfectants after 2 h of treatment. Bleach (sodium hypochlorite) at 6% solution induced 92.7 % inactivation of *C. parvum* oocysts after 2 h. Thermally treated oocysts (56 and 70°C for 20 min) demonstrated complete inactivation, whereas at 38°C no inactivation was observed. Application of Neopredisan® 135- 1 and Aldecoc® TGE (4% for 2h) as recommended according to the current guidelines stipulated by DVG (German Veterinary Society) consistently inactivated more than 99.5% of oocysts. The suggested cc-qPCR method appeared to be suited for standardized testing of inactivation measures, particularly for evaluation of chemical disinfectants and thus cc-qPCR is proposed as an alternative to the established chicken infectivity model for *E. tenella* for testing anticoccidial disinfectants. A minimum inactivation of 99.5% in cc-qPCR model is claimed as a suitable threshold for certification of chemical products for disinfection of coccidia oocysts.

Keywords: *C. parvum*, cell culture infectivity, quantitative PCR, disinfectants efficacy

1. Introduction

Cryptosporidium parvum is a coccidian parasite that affects a wide range of hosts including humans. Infected hosts shed oocysts that are transmitted to new hosts. In general, coccidian oocysts are resistant to environmental conditions and against many disinfectants (Fayer et al., 1996; Barbee et al., 1999). Hygienic measures in terms of cleaning and disinfection are essential to reduce the infection load in the environment. Methods such as in vitro inhibition of sporulation, lysis test, assessment of excystation and vital staining are performed to test for viability of coccidia oocysts but they have limitations (Black et al., 1996; Bukhari et al., 2000) as these tests do not allow reliable estimation of infectivity of oocysts. For instance, excystation and vital dye stain are not able to

predict the efficacy of UV and ozone treatment (Bukhari et. al., 1999, 2000) on *C. parvum*. Sporulation inhibition is not applicable to *Cryptosporidium* as oocysts sporulate endogenously (Rommel, 2000). Oocyst lysis may overestimate inactivation because released sporozoites may still be infective. Furthermore, inactivation estimated from excystation and lysis of oocysts do not reflect in vivo infectivity (Bukhari et al., 2000). Studies on oocyst infectivity in animal infection models after disinfection have been reported for *Isospora* (Straberg and Daugschies 2007), *Toxoplasma* (Hitt and Filice, 1992; Garcia et al., 2006; Wainwright et al., 2007; Dumetre et al., 2008), *Cryptosporidium* (Korich et al., 1990; Joachim et al., 2003) and *Eimeria* (Daugschies et al., 2002). The chicken infection model for *Eimeria tenella* oocysts was standardized for routine screening of anticoccidial disinfectants (Daugschies et. al., 2002) and was approved by DVG (German Veterinary Society) as a standard test required to certify anticoccidial efficacy of disinfectants. Currently disinfectants which lead to more than 95% reduction of oocyst infectivity in the animal model are approved by DVG. However, infectivity testing in animal models is costly and laborious compared to in vitro studies and related to ethical concerns. Studies on infectivity of coccidia including *Toxoplasma gondii* have been performed in cell culture (Bunetel et al., 1995; Evans et al., 1999). *Cryptosporidium parvum* demonstrates many developmental attributes common to enteric coccidia (O'Donoghue 1995). In vitro culture of *C. parvum* was found to suitably reflect the ability of oocysts to cause infection in animals (Hijjawi et al., 2001; Shin et al., 2001; Rochelle et al., 2002). Real time PCR has been applied for quantitative assessment of development of *Cryptosporidium* in a cell culture system (Shahiduzzaman et al., 2009a; Shahiduzzaman et al., 2009b). The present study was aimed to establish whether combination of cell culture and quantitative PCR (cc-qPCR) is suited to replace the currently used chicken model for *E. tenella* to screen anticoccidial disinfectants.

2. Materials and methods

2.1. Parasite and inactivating agents

A field strain of *C. parvum* was maintained through passage in neonatal calves. Oocysts were isolated and purified by established methods (Najdrowski et al., 2007) and stored in phosphate buffered saline (PBS, pH 7.4) with streptomycin (200 µg/ml) and amphotericin B (5 µg/ml) at 4°C. Medium was replaced by fresh stock solution every month. Oocysts of less than 3 months of age were used in all experiments. The DVG approved products (p-Chlor-m-cresol) Neopredisan® 135- 1(Menno Chemie, Norderstedt, Germany) and Aldecoc® TGE (EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany) were used as disinfectants. Other products not approved as anticoccidial disinfectants like Aldecoc® XD (p-Chlor-m-cresol, EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany), IGAVET® FF spezial (aldehyde, COS OHLSEN Chemie & Gerätevertrieb GmbH, Geltorf-Esprehm, Germany) and KokziDes® (Arthur Schopf Hygiene GmbH & Co. KG, Neubeuern, Germany), a disinfectant not yet commercialized, and bleach (sodium hypochlorite), were also tested.

2.2. Oocysts disinfection

All disinfectants were diluted in fresh tap water to 1.1x of final concentration before use. 1×10^6 oocysts were transferred to a 1.5 ml centrifuge tube and washed twice with PBS to remove streptomycin and amphotericin present in the stock solution. The oocysts were resuspended in 100 µl of tap water and 900 µl of the 1.1x concentration of disinfectant was added so that the oocysts were incubated at the final washing concentration of disinfectant. Oocysts were treated in separate 1.5 ml tubes for each concentration. The control was

incubated in tap water. After disinfection at room temperature, the treated oocysts were washed thrice in PBS. Oocysts were counted and density of oocysts was set to 5 x 10^4/200 µl for cell culture inoculation. The oocyst suspension was controlled under microscope for excystation, destruction or morphological changes occurring as a consequence of disinfection treatment.

2.3. Cell culture infection

A human ileocaecal adenocarcinoma (HCT-8) cell line was used for in vitro cultivation of *C. parvum* as described (Shahiduzzaman et al., 2009a). About 2 x 10^5 HCT-8 cells were seeded into each well of 24 well culture plates in growing medium 24 h before inoculation with oocysts. The oocysts (5 x 10^4 per well) were excysted on the confluent monolayer for 3 h at 37°C with 5% CO_2 allowing sporozoites to invade the HCT-8 cells. Monolayers were washed with growing medium to remove the unexcysted, empty or inert oocysts and unbound sporozoites. The infection was maintained for 48 h post inoculation (h p.i.). Control cultures were incubated with untreated oocysts, aldehyde treated oocysts, heat inactivated oocysts or no oocysts. A series of fresh oocyst dilutions (10 to 1 x 10^5) were used to estimate the overall sensitivity of the cell culture infection assay and also for generating a standard curve to calculate the infectivity of oocysts. A dilution series of heat inactivated oocysts (70°C for 1 h) were used to determine the size of the oocyst inoculum at which complete elimination of extracellular parasite material is reliably achieved.

2.4. Preparation of DNA

At the end of the incubation period, growing medium was removed and monolayers were washed thrice with 1 ml of sterile PBS (pH 7.4). Each monolayer was treated with 200 µl of accutase (PAA Laboratories GmbH, Coelbe, Germany). Subsequently, the cells were collected to 1.5 ml tube and centrifuged at 4500 x g for 5 min. The supernatant was gently removed by aspiration and the pellet was resuspended in 200 µl PBS (pH 7.4). DNA from each sample was isolated according to the instructions for blood and body fluid spin of the QIAamp® mini kit (Qiagen, Hilden, Germany). The extracted DNA was eluted in 100 µl of elution buffer supplied with the kit and the elutate was stored at -20°C if not used immediately.

2.5. Real time TaqMan assay

The assay was performed with the primers and probe as described previously (Shahiduzzaman et al., 2009a). In brief, amplification reactions of 25 µl volume contained 1x AB gene master mix (ABgene mastermix, AB gene limited, Surrey, England); 300 and 900 nM forward [CP_hsp70_fwd (2219-2246) 5´-aactttagctccagttgagaaagtactc-3´] and reverse [CP_hsp70_rvs (2336-2362) 5´-catggctctttaccgttaaagaattcc-3´] primers (each 25 µM, Biopolymer, Ulm, Germany), respectively; 200 nM TaqMan probe (HSP_70_SNA 5´-aatacgtgtagaaccaccaaccaatacaacatc-3´) (25 µM, Biopolymer, Ulm, Germany) and 30 nM ROX as a reference dye (1 mM, AB gene). An aliquot of 3 µl template was used in each reaction tube. The reaction was run in a Mx3000P system (Stratagene®, La Jolla, USA) under the following conditions: 95°C for 15 min (enzyme activation), 40 cycles at 95°C for 15 s (initial denaturation) and 60°C for 1 min (annealing and extension of primers). Data represent the mean of 3 replicates with an acceptable standard deviation of less than 0.50.

2.6. Quantitative analysis

The sample DNA copies were obtained from a standard curve (calibration curve) generated automatically by plotting the log of the initial template DNA copy numbers against the Ct generated (reported from fluorescence signals in qPCR reactions) for each dilution. As the

size of the oocyst inoculum increased, the Cycle threshold (Ct) values linearly decreased corresponding to increase of the rate of infection as determined from the number of parasite DNA copies. Based on the sample DNA copies the percent inactivation was usually calculated by using the formula [Number of DNA copies for control – DNA copies for treated oocysts) / DNA copies for control] x 100.

The cell culture dose response calibration curve was generated using MS Excel (Microsoft Corp., WA, USA) by plotting the log initial DNA copies calculated from individual Ct values, to the log inocula in each experiment with the equation $y = mx + b$; where x is log oocyst dose, y is log number of DNA copies (corresponding to number of oocysts excretion), m is slope that reflects infection rate and b is intercept that reflects percent infectivity. Similar to the reference chicken model the number of oocysts remaining infective (infective dose = ID) was calculated as follows:

[(ID value for untreated control oocysts – ID value for treated oocysts / ID value for untreated control oocyst] x 100

All data were statistically analyzed using GraphPad Prism® version 5.0 (GraphPad software Inc., La Jolla, CA, USA). Difference in the slope and intercept among the calibration curves were determined using a linear regression model. *t* test was performed to determine the difference in infectivity between the chicken infection and cell culture infection models. Non-linear regression using a sigmoidal model was used to calculate the 50% infectious dose (ID_{50}). Pearson correlation was used to show the relationship between two variables.

3. Results

3.1. Cell culture assay sensitivity

Ct values generated against standard dilution of oocysts provided a significant ($p < 0.05$) linear correlation (r^2) at 24 h p.i. ($r^2 = 0.996$) and 48 h p.i. ($r^2 = 0.992$) with lower Ct after the longer incubation period (Fig. 1.). For 100 oocysts, inconsistent fluorogenic signals (1 of 3 replications) in qPCR were observed at 24 h p.i. but signals were constant (3 of 3) with a mean Ct value of 35.08±0.11 at 48 h p.i. A similar inconsistency was observed for samples inoculated with 10 oocysts at 48 h p.i. and no signals were recorded for this inoculation level at 24 h p.i., indicating a lower detection limit of 100 oocysts after 24 h p.i. and 10 oocysts after 48 h p.i. Inoculation with up to 1 x 10^5 heat inactivated oocysts did not produce any signal, showing that extracellular oocysts are successfully removed from monolayers by the applied washing procedure (data not shown).

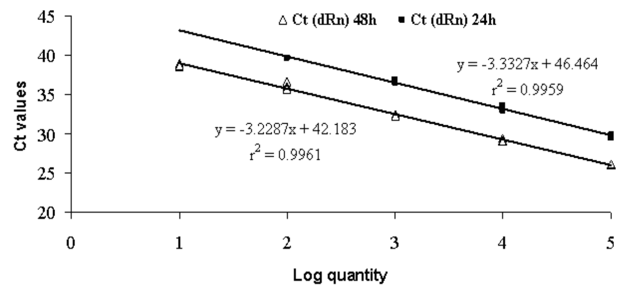

Fig. 1. Regression curve calculated for Ct of cell cultures infected with serial dilution of *Cryptosporidium* oocysts ($p < 0.001$).

3.2. Oocysts inactivation

Microscopic investigation of treated (disinfectants or temperature) *C. parvum* oocysts did not reveal any excystation or destruction of oocysts irrespective of disinfectant concentration and time. The cell culture incubation with oocysts treated by a range of concentrations of Neopredisan® 135-1 demonstrated a dose dependent significant ($p < 0.001$) reduction of viability of oocysts ($r^2 = 0.929$, fig. 2). At 4% concentration infectivity was reduced by 99.91, 99.91 and 98.50% after 2 h of incubation with Neopredisan® 135- 1,

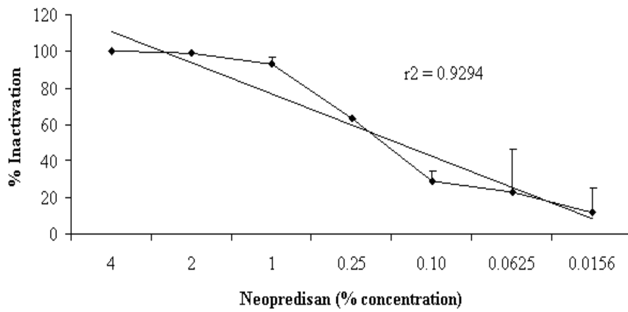

Fig. 2. Percent inactivation of oocysts treated with different concentrations of Neopredisan® 135- 1 for 2 h. Error bar represents standard deviation of mean.

Table 1
Inactivation measurement of *C. parvum* oocysts in cell culture model

Oocysts treatment	(%inactivation ± SD)
Thermal (20 min)	
70°C	100 ± 0.01
56°C	100 ± 0.01
38°C	0 ± 0.25
Neopredisan® 135- 1 (2 h)	
1%	93.93 ± 2.38
2%	99.21 ± 0.57
3%	99.11 ± 0.73
4% [a]	99.91 ± 0.08
Aldecoc® TGE (2 h)	
1%	89.19 ± 1.29
2%	98.47 ± 0.57
4% [a]	99.91 ± 0.05
Aldecoc® XD (2 h)	
2%	79.11 ± 3.74
4%	98.50 ± 0.36
IGAVET FF spezial (2 h)	
2%	- 0.01 ± 0.01
4%	- 0.16 ± 0.22
Bleach (2 h)	
3%	73.57 ± 5.78
4%	83.09 ± 1.86
6%	92.7 ± 1.39

SD is standard deviation of mean of sample replicates from at least two experiments.
[a]Concentration approved for anticoccidial disinfection according to DVG guidelines.

Aldecoc® TGE and Aldecoc® XD, respectively. Application of 2% Neopredisan® 135- 1 for 2 h demonstrated 99.21% reduction of infectivity whereas 98.47% reduction was observed for Aldecoc® TGE at similar conditions. The effect of Aldecoc® XD at 2% for 2 h was lower with a mean inactivation of 79.11%. IGAVET FF spezial, an aldehyde containing product, was completely unable to inactivate *C. parvum* oocysts at any concentration used. *Cryptosporidium parvum* oocyst exposure to 6, 4 or 3% bleach solution for 2 h resulted in 92.7, 83.09 and 73.57% mean inactivation, respectively. Applying a range of temperature to inactivate C. *parvum* oocysts resulted in a complete inactivation at 56 and 70°C for 20 min whereas no inactivation was found at 38°C (Table 1). KokziDes® demonstrated a dose and time dependent reduction of oocysts viability by cc-qPCR. Application of 4, 3, 2 and 1% concentration of KokziDes® resulted in a mean reduction of 99.87, 99.86, 99.85 and 86.02 % viability of oocysts, respectively, after 2 h of incubation (Table 2).

The individual log dose-response linear association provided four calibration curves (equations) from four replicate sets (different oocyst lots of same size) of cell culture experiments termed as calibration curve- A, B, C, and D; [A, ($y = 0.8173x + 1.1209$), correlation- $r^2 = 0.9948$; B, ($y = 0.9356x + 0.5266$), $r^2 = 0.9961$; C, ($y = 0.9429x + 0.7175$), $r^2 = 0.9954$; D, ($y = 0.9492x + 0.9937$), $r^2 = 0.9953$]. Regression analysis of log dose- response provided no significant differences between slope of calibration curve B, C and D ($p = 0.883$), indicating the homogeneity of experimental conditions and of oocyst lots regarding cell culture infectivity. The slope of calibration curve A differed significantly ($p < 0.0001$ values). The differences in mean inactivation values between the two calculation systems (calculation by comparing DNA copies and calculation on the basis of ID values) provided a minimum level variability (< 0.5% CV) indicating homogeneity of results obtained from both calculations (Table 3).

Table 2
Effects of KokziDes® on infectivity of *C. parvum* oocysts in cell culture model

Concentration	4%		3%		2%		1%	
Hour of incubation	1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h
Trial 1 (% inactivation)	99.62	99.84	99.50	99.82	99.08	99.78	69.02	87.02
	99.67	99.89	99.31	99.89	99.20	99.90	69.02	85.82
	99.61	99.90	99.38	99.88	99.02	99.87	66.33	85.21
Trial 2 (% inactivation)	99.63	99.77	99.47	99.74			56.41	90.26
	99.57	99.83	99.51	99.79	-	-	55.35	91.50
	99.51	99.81	99.47	99.87			55.73	91.32

Individual data represents sample replicate within the trial

Table 3
Comparison of percent inactivation data in the cc-qPCR model for chemical disinfectants at 4% concentration and 2 h of incubation

Disinfectant	[a](%inactvation) by comparing DNA load	[b](% inactivation) by comparing ID values				[c](%CV)
		Calibration curves				
		A	B	C	D	
Neopredisan® 135- 1	99.8	99.95	99.87	99.86	99.86	0.05
	100	99.98	99.94	99.94	99.94	0.03
	99.92	99.98	99.95	99.95	99.95	0.02
Aldecoc® TGE	99.98	100	99.99	99.99	99.99	0.01
	99.86	99.96	99.9	99.9	99.89	0.03
	99.9	99.97	99.93	99.92	99.92	0.02
Aldecoc® XD	98.06	99.15	98.45	98.4	98.36	0.37
	98.5	99.42	98.88	98.84	98.8	0.30
	98.95	99.6	99.19	99.16	99.14	0.22

Individual data represents the mean value of sample replicates at each trial
[a]Percent inactivation = [Number of DNA copies for control – DNA copies for treated oocysts) / DNA copies for control] x 100
[b]Percent inactivation = [(ID value for untreated control oocysts – ID value for treated oocysts) / ID value for untreated control oocysts] x 100
[c]coefficient of variation between [a] and [b]

4. Discussion

For the purpose of certification of disinfectants reproducibility of test results is crucial (Marx, 1995). The cc-qPCR applied for assessment of drugs against *Cryptosporidium* (Shahiduzzaman et al., 2009a; Shahiduzzaman et al., 2009b) along with some modifications appears also to be suitable for quantification of oocyst inactivation and thus may replace the animal infection model currently used. Cell culture infection with ten fold serial dilutions of viable oocysts (1 x 10^5 to 10) displayed a significant correlation to qPCR results indicating sensitivity and reliability of cc-qPCR and provides means for reliable quantification of oocyst inactivation. Consistent and reliable detection of infection in monolayers infected with an extremely low

dose is increased by longer incubation reflecting the longer time that it takes the parasite to multiply to a detectable level. This is in accordance with the study of Keegan et al. (2003). The cell culture standard curve generated in our assay is compatible with the plasmid standard used in previous studies (Fontaine and Guillot, 2002; Shahiduzzaman et al., 2009a).

Table 4: Comparison of percent inactivation data for the infectivity models

Disinfectant	Treatment (2 h)	Percent oocysts inactivation	
		[a]Chicken model for *E. tenella*	Cell culture model for *C. parvum*
[b]Neopredisan® 135-1	3%	92.9	99.40
	4%	96.8	99.93
	3%	90.6	99.11
	4%	95.2	99.91
[b]Aldecoc® TGE	4%	95.5	99.91
[c]KokziDes®	3%	95,5	99.86
	4%	99,93	99.87

[a]data from animal experiments (according to DVG guideline) routinely performed to test anticoccidial disinfectants at institute of Parasitology, Universität Leipzig , Germany
[b]DVG approved disinfectants (recommended concentration 4% for 2 h)
[c]product not yet commercialized

The suitability and reproducibility of the cc-qPCR assay for inactivation studies were also tested by using thermally treated oocysts. The results were comparable to those obtained by Najdrowski et al. (2007). The current assay was further validated by testing anticoccidial disinfectants approved by DVG (update list published each year; e.g. Böhm, 2000). The suitability of Neopredisan® 135- 1 as an anticoccidial disinfectant has been well documented by a variety of methods. However efficacy has been found to vary depending on the concentration and time of oocyst exposure to the disinfectant. In the present study Neopredisan® 135- 1 resulted in a significant dose dependent reduction of infectivity and was found to be consistently effective over 99.5% at 4% concentration for 2 h. This is similar to the in vitro results observed by Eckert (2001) and Najdrowski et al. (2007). Lower effectiveness of Neopresdisan® at 2% and 3% concentration for 2 h was in accordance with the in vivo results reported by Joachim et al. (2003). Bleach solution at 6% concentration resulted in a reduction of 92.7% infectivity. A significant reduction of lesion scores in mice was observed by Fayer (1995) after incubation of oocysts with 5.25 or 2.63% of bleach, however, variation of estimation of oocysts is to be expected when different methods are applied. No inactivation was found when oocysts were treated with Aldehyde (IGAVET FF® Special) in our assay. Inactivation determined by both the in vitro (Table 2) and the in vivo model (Table 4) for KokziDes® (3% concentration for 2 h) exceeded the threshold value of 95 % (in vivo) and 99.5 % (in vitro). Thus KokziDes® was found to be active against *C. parvum* and *E. tenella* and parallel testing of the product in both models showed that the threshold value of 99.5 % for *C. parvum* appears suitable.

The main objective of this study was to analyse whether cc-qPCR has the potential to replace the established chicken infection assay with *E. tenella* for standardized testing and certification of disinfectants. The infection responses in cell culture for *C. parvum* were compared with the infection response in chicken model for *E. tenella* (Daugschies et al. 2002). Regression analysis of log dose-response linear associations from cell culture infection with four oocysts lots of same size revealed no significant differences between slopes (reflecting no significant variation in infection rate) but intercept differed to some extend reflecting lot to lot variation in infectivity, which is supported by the results of Slifko et al. (2002). However, these differences did not give a remarkable effect on calculation of infectivity. The variability in infectivity has to be considered by calculating

results in comparison to control cultures within each experiment. Inter or intra experiment variation in both the infection rate (slope) and percent infectivity (intercept) are often observed in animal model (Daugschies et al., 2002; Rochelle et al., 2002; Slifko et al., 2002). The advantage of the cc-qPCR model over these limitations of animal models are well defined experimental conditions that can be fully standardized and do not underlie individual variations of animals.

Linear correlation between inoculation dose and infection response is a suitable basis to back calculate the actual number of infective oocysts after disinfection. Percent inactivation calculated as a difference of DNA copies was not significantly different from the percent inactivation calculated on basis of ID values. This study along with previous reports confirms that the dose response in cc-qPCR appears to be at least as suitable for disinfection testing as the animal assay. A significant and reproducible dose response relationship in cell culture is most important to obtain reliable data on disinfectants efficacy. Comparison of the $\log_{10}$ normalized ID_{50} (50% of infectious dose) values calculated for both models by *t*- test suggests that *C. parvum* infectivity in cell culture is statistically different from *E. tenella* infectivity in the chicken model ($p<0.05$). However, the sensitivity in both models correlated significantly ($r^2 = 0.9795$; $p<0.05$). Relative inactivation calculated for *C. parvum* in cc-qPCR was higher than in the chicken model for *E. tenella* (Table 4) at the respective inactivation conditions. Variations in effects of a disinfectant between these two models are attributable to structural and biological differences of oocysts of *C. parvum* and *E. tenella* and the method of detection/quantification. In the chicken model a minimum of 95% inactivation is claimed as the threshold for certification of a product to be suitable for anticoccidial disinfection. For cc-qPCR the threshold value has to be set to a higher relative value to preserve the strict conditions for assessment of disinfectants. It is shown in this study that all products that proved efficient in the chicken model exceeded the proposed 99.5% threshold in CC-qPCR whereas disinfectants without sufficient efficacy on *E. tenella* remained below this level. Based on the current results a minimum of 99.5% inactivation is proposed for certification of a product to be sufficiently effective. Application of the recommended concentration (4% for 2 h) approved by DVG for Neopredisan® 135- 1 and Aldecoc® TGE on basis of the chicken infection assay consistently exceeded the postulated threshold value of 99.5% postulated for cc-qPCR. Thus cc-qPCR is considered to be a suitable alternative for the chicken infectivity model for testing and approval of anticoccidial disinfectants.

In conclusion, cc-qPCR is simple and robust for routine screening of disinfectants against *C. parvum*. This model for *C. parvum* can be considered as equivalent to the chicken infectivity model for *E. tenella* regarding linear association of the log dose-response curve and sensitivity. It is proposed that consistent effects of a disinfectant for each of at least three replicate samples must be ≥ 99.5% inactivation to certify a product as a suitable anticoccidial disinfectant.

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4 Summary of results and discussion

The optimized cell culture infection provided reliable and reproducible estimation of parasite multiplication in culture. The efforts for conducting the studies led to a better understanding of *C. parvum* cell culture infectivity and analysis. The current studies serve the purpose for the quantitative evaluation of new potent drugs and disinfectants under *in vitro* conditions. The proposed method meets the urgent need for a rapid, reliable and workable experimental model for evaluation of drugs and disinfectants against cryptosporidia and may replace animal models such the chicken infection model that is mandatory for testing and certification of anticoccidial disinfectants in Germany.

4.1 Standardized cell culture infectivity assay (Publication 1, 2 and Manuscript 3)

The initial seeding of 2 x 10^5 HCT-8 cells/well (24 well microplate) and of 1 x 10^6 HCT-8 cells/well (6 well microplate) were best suited for subsequent infection with 5 x 10^4 and 2.5 x 10^5 oocysts, respectively. Under optimized cell culture infection conditions a period of 48 h incubation delivered maximum parasite numbers without any alteration of host cell monolayers and enabled reliable (accurate and reproducible) assessment of parasite multiplication in culture. Thermally inactivated oocysts (less than 1 x 10^5) did not produce any signal in qPCR indicating complete removal of inert or unexcysted oocysts by the applied washing procedure.

Monolayers were seeded with serial dilutions of oocyst suspensions (10^6 to 1) and subsequent qPCR resulted in a linear correlation of the log of the initial template DNA copy numbers against the Ct generated for each dilution. Infection with at least 10 oocysts was detectable after 48 h p.i. and with 100 oocysts after 24 h p.i. The detection limit was 10^2 copies for plasmid DNA or 25 oocysts corresponding to 100 sporozoites. Assay quality was similar whether cell culture, plasmid DNA or infected control culture DNA was used for calibration and independent of size or type of the culture plate (24 or 6 wells) and range of template DNA (120-700 ng/reaction) indicating the flexibility and reliability of the assay. The incubation period of infected cultures may influence sensitivity of detection and subsequently the end point results, however, selection of the appropriate time depends on e.g. the period expected for the applied drugs to exert the maximum effect during the course of development. Longer incubation increases the chance to detect

successful infection from a low number of viable oocysts and is needed for assessment of inactivation measures e.g. application of disinfectants.

4.2 *In vitro* drug screening (Publication 1)

The toxicity of the applied drugs was assessed by microscopical alteration of the monolayers and individual HCT-8 cells. The results reported here for monensin (98% parasite growth inhibition at 0.144 µM after 27 h p.i. without toxicity to host cells) supports results of previous studies (MC DONALD et al. 1990, ARMSON et al. 1999, NAJDROWSKI et al. 2007). Halofuginone is used for treatment of cryptosporidiosis in calves, however, has limited efficacy (LEFAY et al. 2001, JOACHIM et al. 2003). *In vitro* efficacy was very clear (99% inhibition at 1 µM after 27 h p.i.) which is in accordance with previous reports (NAJDROWSKI et al. 2007). Inhibition of parasite proliferation was increased after 15 h p.i. and was much more pronounced at 27 and 45 h p.i. Dose dependent significant ($P \leq 0.05$) reduction of parasite multiplication was recorded for each of the applied drugs. A maximum of 98% inhibition was found after application of 24.5µM miltefosine at 45 h p.i. The relative parasite DNA copy number calculated after the drug exposure compared to the initial reference value (3 h p.i., before administration of drug) demonstrated to 97% (monensin), 99% (halofuginone) and 96% (miltefosine) reduction in multiplication compared to the non treated control at 27 h p.i. Comparing the level of parasite DNA copies over the incubation period with the reference value at 3 h p.i. (before application of drugs) the conclusions on efficacy and mode of action of the applied drugs (parasitocidal or parasitostatic) are justified. Evaluation of miltefosine, an anti-tumor and anti-leishmanial agent (SUNDAR et al. 2000), provided a lower level of efficacy than for monensin and halofuginone. Nevertheless, anticryptosporidial potency of miltefosine can be deduced from the *in vitro* data and thus this drug may be further evaluated for suitability in the control of cryptosporidiosis.

4.3 *In vitro* effects of curcumin on *C. parvum* (Publication 2)

The initial evaluation of toxicity by MTT suggests that curcumin at a concentration of up to 50 µM over 24 h was not toxic to HCT-8 cells and accordingly pre-treated cells supported subsequent infection and development of *C. parvum*. Curcumin displayed dose dependent inhibition of multiplication of *C. parvum* and application of fresh curcumin preparation every 12 h greatly

increased the effectiveness over the incubation period. The resulting IC_{50} value of 13 µM indicated that *C. parvum* multiplication inhibition by curcumin was more profound than previously reported for *Plasmodium* (CUI et al., 2007), *Giardia* (PEREZ-ARRIAGA et al. 2006) and *Leishmania* (KOIDE et al. 2002). Discontinuation of curcumin treatment with 50 and 25 µM after 24 h did not allow resumption of *C. parvum* multiplication in cell culture. Curcumin inhibits (65% at 200 µM for 3 h) the invasion of sporozoites to HCT-8 cells. The molecular mechanisms involved are yet to be identified. However, curcumin interaction with biomolecular or cellular mechanisms reported for other apicomplexan parasites (CUI et al. 2007, SAFFER et al. 1989, SAFFER and SCHWARTZMAN 1991) might also affect *C. parvum* infectivity and development.

4.4 *C. parvum* oocysts inactivation studies (Manuscript 3)

The applied chemical disinfection or thermal treatment did not induce excystation or destruction of exposed oocysts, and thus no loss of oocysts due to treatment was observed before being inoculated into cell culture. Application of oocysts treated by a range of concentrations of Neopredisan® 135-1 resulted in a dose dependent reduction of oocyst viability and the results were similar to those obtained by ECKERT (2001), NAJDROWSKI et al. (2007) and JOACHIM et al. (2003). Thermal treatment (38, 56 and 70°C for 20 min) of the oocysts showed results that are consistent with those obtained by NAJDROWSKI et al. (2007).

The infection responses in cell culture for *C. parvum* were compared with the previously reported infection response in the chicken model for *E. tenella* (DAUGSCHIES et al. 2002). In four trials the calibration curve calculated from parasite DNA copies (as infection response) in cultures infected with serial dilutions of a suspension of untreated oocysts (after logarithmic transformation of values) showed a clear linear association ($r^2 = 0.9948$ to 0.9961) which provided a suitable basis to back calculate the actual number of oocysts remaining infective after disinfection. Use of oocyst lots less than 3 months old resulted in similar calibration curves (log dose-log DNA copies) indicating homogenous experimental conditions and neglectable variation in cell culture infectivity. Lot to lot variability in infectivity did not give a remarkable effect on calculation of inactivation because the variability in infectivity is considered by calculating inactivation in comparison to control cultures within each experiment.

Comparison of the $\log_{10}$ normalized ID_{50} (50% of infectious dose) values calculated for both models by *t*- test suggests that *C. parvum* infectivity in cell culture is statistically different from *E. tenella* infectivity in the chicken model ($p<0.05$). However, the sensitivity in both models correlated significantly ($r^2 = 0.9795$; $p<0.05$). Results obtained after anticoccidial disinfection (Neopredisan® 135-1 and Aldecoc® TGE, DVG listed products published each year; e.g. BÖHM 2000) at the recommended concentration and time (4% for 2 h) provided effects that consistently exceeded 99.5% inactivation. Therefore, 99.5% inactivation is claimed as a suitable threshold value for certification of a product to be sufficiently effective against coccidia. Higher inactivation was observed in cell culture model for *C. parvum* than in the chicken model for *E. tenella* at the respective inactivation conditions. Variations in effects of a disinfectant between these two models are attributable to structural and biological differences of oocysts of *C. parvum* and *E. tenella.* Aledcoc® XD, bleach as well as IGAVET® FF spezial did not exceed the threshold. These disinfectants are not considered active against cryptosporidia which is in accordance with the results of cc-qPCR. Inactivation determined by both the in vitro and the in vivo model for KokziDes® (3% concentration for 2 h) exceeded the threshold value of 95 % (in vivo) and 99.5 % (in vitro). Thus KokziDes® was found to be active against *C. parvum* and *E. tenella* and parallel testing of the product in both models showed that the threshold value of 99.5 % for *C. parvum* appears suitable.

5 Conclusion

The optimized cell culture infection and quantification assay is suitable for reliable and reproducible estimation of *C. parvum* oocyst infectivity. The cc-qPCR assay was applied successfully to evaluate anticryptosporidial drugs and disinfectants. Miltefosine and curcumin showed anticryptosporidial activity in this assay, however, the suitability of these drugs for treating cryptosporidiosis need to be further tested in animal models. The standardized cc-qPCR may replace the chicken infection model for *E. tenella* as a standard test to certify anticoccidial efficacy of disinfectants.

6 Summary

Md. Shahiduzzaman

Combination of cell culture and quantitative PCR (cc-qPCR) for assessment of efficacy of drugs and disinfectants against *Cryptosporidium parvum*

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Cryptosporidium parvum is an obligatory intracellular parasitic protist that belongs to the phylum Apicomplexa. Cryptosporidiosis is an infection for which no satisfactory efficient curative treatment is known, especially in immunocompromised individuals. Furthermore, the parasite oocysts show considerable tenacity in the environment. Therefore, new potent drugs along with a simple and reliable experimental model for evaluation of anticryptosporidial measures are urgently needed.

The present studies were undertaken to establish a combined cell culture and quantitative PCR assay (cc-qPCR) to assess efficacy of pharmacological compounds against *C. parvum*. Human ileocecal adenocarcinoma cells (HCT-8) were selected for culture of *C. parvum*. Oocysts were excysted directly on confluent monolayers for infection. After 3 h of incubation the non invasive parasite remains were removed by washing. At the end of the incubation period the cells were harvested and subjected to DNA extraction. Real time PCR was performed to quantify the target parasite DNA (fragments of 70 kDa heat shock protein gene) copy numbers. Each reaction was run in triplicate. A standard curve calculated on the basis of serial dilutions of plasmid DNA or infected control culture DNA was run in each experiment. A series of oocyst suspensions were applied to cell cultures to determine the sensitivity of the cc-qPCR assay and also to generate a calibration curve to calculate the infectivity of oocysts. A dilution series of heat inactivated oocysts (70°C for 1 h) were used to determine the size of the oocyst inoculum at which complete elimination of extracellular parasite material by washing is reliably achieved. The results obtained by the assays were reproducible and the method sensitive with a detection limit of infection with 10 oocysts 48 h post infection (p.i.) and with 100 oocysts 24 h p.i. Percent effects of drugs and disinfectants were enumerated by comparing DNA copies between treated and non treated samples.

The suitability of cc-qPCR for screening of pharmacological compounds was validated by confirming the *in vitro* efficacy of monensin (98.15% ± 1.09 at 0.144 µM) and halofuginone (98.05% ± 0.59 at 25 µM) over the entire incubation period with a dose dependent reduction of parasite multiplication demonstrated 27 h p.i. The inhibition of parasite proliferation by 0.144 µM monensin in the period from 3 h p.i (time defined to represent the initial level of parasite development before drug application) to 27 h p.i. or 45 h p.i. was 97 and 99% respectively, and by 25 µM halofuginone 99% (27 h p.i.). Hexadecylphosphocholine (miltefosine), a new anti-leishmanial compound, was tested against cryptosporidia and provided a maximum of 98% reduction of parasite multiplication at 45 h p.i.

The potential activity of curcumin (extract from the herb *Curcuma longa*) against *C. parvum* was also evaluated by cc-qPCR. Curcumin appeared to be sensitive to degradation after prolonged incubation and the observed inhibition of multiplication of *C. parvum* was significantly increased when medium was replaced by fresh medicated medium after 12 h of exposure. The effects on parasite multiplication (>95% inhibition with IC_{50} value of 13 µM) and on sporozoite invasion (assessed 3 h p.i.; 65% inhibition at 200 µM) suggest that further exploration of anticryptosporidial efficacy of curcumin may be rewarding.

The cc-qPCR was further optimized to analyse inactivation measures directed against oocysts of *C. parvum.* The suitability of the assay for assessment of inactivation measures was confirmed by the reproducible demonstration of effectiveness of cresolic disinfectants at the recommended concentration of 4% and incubation period of 2 h (Neopredisan® 135-1, Menno Chemie, Norderstedt, Germany: 99.91% ± 0.08; Aldecoc® TGE, EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany: 99.91± 0.05) and by using thermally inactivated oocysts (complete inactivation by 56°C and 70°C for 20 min). Based on the *in vitro* results and previously obtained data from the chicken infection model 99.5% inactivation is proposed as a suitable threshold value that needs to be consistently exceeded by a product to be considered efficient. Application of Neopredisan® 135- 1 and Aldecoc® TGE (4% for 2h) consistently inactivated more than 99.5% of oocysts while other disinfectants that are not certified as anticoccidial products like Aldecoc® XD (EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany) and IGAVET® FF spezial (COS OHLSEN Chemie & Gerätevertrieb GmbH, Geltorf-Esprehm, Germany) and bleach (sodium hypochlorite) did not.

It can be concluded that the cc-qPCR method is suited to easily and reliably assess anticryptosporidials *in vitro*. The method demonstrated that miltefosine and curcumin display anticryptosporidial efficacy under the applied conditions. The cc-qPCR is a highly standardized method supposedly appropriate to replace the chicken infection model for *Eimeria tenella* as currently practised for certification of anticoccidial disinfectants according to the guidelines of DVG (German Veterinary Society).

7 Zusammenfassung

Md. Shahiduzzaman

Kombination von Zellkultur und quantitativer PCR (cc-qPCR) zur Prüfung der Wirksamkeit von Arzneimitteln und Desinfektionsmaßnahmen gegen *Cryptosporidium parvum*

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Cryptosporidium parvum ist ein obligat intrazellulärer Protozoe aus dem Stamm der Apicomplexa. Für die Kryptosporidiose gibt es derzeit kein hinreichend effizientes Therapeutikum. Die infektiösen Oozysten weisen eine hohe Tenazität in der Umwelt auf. Aus diesen Gründen ist die Suche nach Wirkstoffen und die Entwicklung eines einfachen und zuverlässigen Prüfmodells für gegen Kryptosporidien gerichtete Maßnahmen dringend notwendig.

Die aktuellen Untersuchungen hatten zum Ziel, ein aus Zellkultur und quantitativer PCR kombiniertes Verfahren zur Prüfung von Wirkstoffen zu etablieren. Humane ileozäkale Adenokarzinomzellen (HCT-8) wurden für die Zellkultur con *C. parvum* ausgewählt. Die Oozysten wurden direkt auf dem konfluenten Monolayer exzystiert und nach 3 h Inkubation die restlichen nicht invasiven Stadien durch Waschen aus den Kulturen entfernt. Nach Abschluß der Inkubation wurden die Zellen geerntet und DNA extrahiert. Die Zielfragmente der parasitären DNA (70 kDa-Fragmente des Hitzeschockprotein-Gens) wurden mittels Echtzeit (*real time*)-PCR quantifiziert. Jede Reaktion wurde als Triplikat durchgeführt. Eine Standardkurve wurde auf Basis serieller Verdünnungen von Plasmid-DNA oder aus infizierten Kontrollkulturen extrahierter DNA für jeden Versuch errechnet. Eine Reihe von Oozystensuspensionen verschiedener Dichte wurde eingesetzt, um die Sensitivität des cc-qPCR festzustellen und eine Eichkurve für die Bestimmung der Infektiosität der Oozysten zu erhalten. Hitzeinaktivierte Oozysten (70 °C für 1 h) wurden verwendet um die höchste Oozystenmenge abzuschätzen, die durch das Waschen der Monolayer sicher und vollständig entfernt werden kann. Die Ergebnisse erwiesen sich als reproduzierbar und die Methode als sensitiv, indem eine Infektion mit 10 Oozysten 45 h post infectionem (p.i.) und mit 100 Oozysten 24 h p.i. nachweisbar war. Die in Prozent angegebene Wirkung von Arzneimitteln und Desinfektionsmitteln wurde durch Vergleich der DNA-Kopienzahl behandelter und nicht behandelter Kulturen bestimmt.

Die Eignung der cc-qPCR für die Prüfung von Pharmaka wurde durch den Nachweis der bekannten In-vitro-Wirkung von Monensin (98,15 % ± 1.09 bei 0,144 µM) und für Halofuginon-Bromid (98,05 % ± 0,59 bei 25 µM) über den Inkubationszeitraum bestätigt, wobei 27 h p.i. eine Dosisabhängigkeit gezeigt werden konnte. Die Inhibition der parasitären Proliferation durch 0,144 µM Monensin im Zeitraum von 3 h p.i. (definiert als Startpunkt zur Quantifizierung parasitärer Entwicklung vor Zugabe der Wirkstoffe) bis 27 h p.i. oder 45 h p.i. lag bei 97 bzw. 99 %.

Halofuginon in einer Dosis von 25 µM inhibierte 27 h p.i. zu 99 %. Hexadecylphosphocholin (Miltefosin), ein Wirkstoff zur Leishmaniose-Behandlung, wurde gegen Kryptosporidien getestet und erreichte 45 h p.i. eine 98 %ige Reduktion der Parasitenvermehrung.

Die potenzielle Aktivität von Curcumin (ein Extrakt aus der Gewürzpflanze *Curcuma longa*) gegen Kryptosporidien wurde ebenfalls mittels cc-qPCR untersucht. Curcumin erwies sich nach längerer Inkubation als offenbar instabil und die festgestellte Inhibition war weit deutlicher, wenn 12 h nach Zugabe des Präparats das Kulturmedium durch frisch präpariertes medikiertes Medium ersetzt wurde. Die Wirkung auf die parasitäre Vermehrung (> 95 % Hemmung, IC_{50} = 13 µM) und auf die Sporozoiten-Invasion (3 h p.i. gemessen; 65 % Hemmung bei 200 µM) lässt weitere Untersuchungen zur Wirkung des Curcumins als sinnvoll erscheinen.

Die cc-qPCR-Technik wurde weiter optimiert, um Inaktivierungsmaßnahmen gegen Oozysten von *C. parvum* zu prüfen. Die Eignung des Modells wurde durch den reproduzierbaren Nachweis der bekannten Effektivität von Kresol-haltigen Desinfektionsmitteln gemäß der empfohlenen Konzentration von 4 % und der Wirkdauer von 2 h (Neopredisan® 135-1, Menno Chemie, Norderstedt, Germany: 99,91% ± 0,08 ; Aldecoc® TGE, EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany: 99,91± 0,05) und den Einsatz von durch Hitze inaktivierten Oozysten (56°C und 70°C über 20 min) belegt. Basierend auf diesen und zuvor im Hühnerinfektionsmodell erhobenen Daten werden 99,5 % Inaktivierung als der Schwellenwert vorgeschlagen, den chemische Produkte sicher überschreiten müssen, damit sie als ausreichend wirksam gelten können. Neopredisan® 135- 1 und Aldecoc® TGE (4%, 2h) zeigten diese Wirkung in allen Versuchen, wohingegen andere Desinfektionsmittel wie Aldecoc® XD (EWABO Chemikalien GmbH & Co. KG, Wietmarschen, Germany) und IGAVET® FF spezial (COS OHLSEN Chemie & Gerätevertrieb GmbH, Geltorf-Esprehm, Germany), die nicht als kokzidienwirksam gelten, sowie Natriumhypochlorit diesen Schwellenwert nicht überschritten.

Die cc-qPCR ist eine einfache und zuverlässige Methode, um Wirkstoffe gegen Kryptosporidien *in vitro* zu testen. Es konnte gezeigt werden, dass Miltefosin und Curcumin unter diesen Bedingungen eine Wirkung gegen Kryptosporidien haben. Die Methode ist gut standardisierbar und möglicherweise geeignet, um die gegenwärtig erforderliche Prüfung von Desinfektionsmitteln auf Kokzidienwirksamkeit im Hühnerinfektionsmodell mit *Eimeria tenella* nach der Richtlinie der DVG zu ersetzen.

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