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Short communication

Bovine *Cryptosporidium parvum* field isolates differ in cytopathogenicity in HCT-8 monolayers

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ABSTRACT

Suckling calves are prone to *Cryptosporidium* infection. The variable degree of clinical disease is influenced by keeping conditions and immune status of the host, but diversity of isolate virulence may also contribute. The aim of the current study was to evaluate the cytopathogenic effects of 26 *C. parvum* field isolates by using a MTT assay in HCT-8 cell monolayers. Cell viability of monolayers inoculated with oocysts of the field isolates varied considerably with values of 17.7% (± 5.1%) to 99.5% (± 7.1%). A standard deviation of 18.6% was detected for cell viability of the in house reference strain, which were tested alongside in every assay. Field isolates were grouped in three categories of cytopathogenicity. Probably the length of storage has an effect on the level of the cell destruction category detected post infection *in vitro*. The applied tool may help to better understand the variable course of cryptosporidiosis in the field.

1. Introduction

Cryptosporidium is an apicomplexan parasite that infects a wide range of humans and animals all over the world (Ryan and Hijjawi, 2015). The clinical course in human depends on the immune status of the host and may be subclinical, moderate or even life-threatening (Collinet-Adler and Ward, 2010). In developing countries *Cryptosporidium* is the second most common pathogen in infants with diarrhoea (Kotloff et al., 2013).

In veterinary medicine *C. parvum* is one of the most important enteropathogens in pre-weaned calves (Cho et al., 2013). The diversity of virulence among field isolates may be of relevance regarding the variable severity of cryptosporidiosis in calf herds as suspected by Fayer and Ungar (1986). The clinical manifestation of cryptosporidiosis arises from the ability of the parasite to invade host enterocytes which results in cell death and cell damage (Sayed et al., 2016). The reduction of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) by living cells is established and commonly applied to quantify the viability of *in vitro* cultured cells (Vistica et al., 1991). In the current study MTT was used to evaluate the viability of human ileocecal adenocarcinoma (HCT-8) cell monolayers infected by different *C. parvum* field isolates.

2. Materials and methods

The in house reference strain (*gp60* subtype IIaA15G2R1) was isolated from a diarrhoetic calf in Köllitsch, Germany, in 2015. Oocysts are passaged in neonatal calves at least every three months. Field isolates of *C. parvum* were obtained by faecal samples from calves of 26 cattle farms located in Saxony (n = 25) and Brandenburg (n = 1), Germany, which were visited in the context of an epidemiological survey (Holzhausen et al., 2019). Faeces were examined microscopically for *Cryptosporidium* oocysts using carbolfuchsin-stained faecal smears (Heine staining). Based on the average number of oocysts in 10 randomly selected fields of vision, the intensity of excretion was evaluated semi-quantitatively according to Holzhausen et al. (2019): 0 = negative; 1 = 0.1–1 oocysts per field; 2 = 1.1–10 oocysts per field; 3 = 10.1–20 oocysts per field; 4 = 20.1–30 oocysts per field; 5 = more than 30 oocysts per field. For further clinical examination of the sampled calves a two tier score system for eight parameters was used: raising: 1 = autonomous, 2 = with help; behavior: 1 = normal, 2 = apathetic; eyes: 1 = clear, 2 = dull; flanks: 1 = filled, 2 = empty; coat: 1 = shiny, 2 = lusterless; dehydrated: 1 = no, 2 = yes; fever: 1 = no, 2 = yes; diarrhoea: 1 = no; 2 = yes. A calf was considered dehydrated when the skin fold of the upper eyelid elapsed after more than three seconds. Fever was defined as an internal body temperature > 39.5 °C. Diarrhoea was defined as a mushy, soupy or watery faecal consistency.

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An overall clinical score (OCS) was calculated for each calf on each visit by summing up score values for each of the above parameters resulting in OCS of between 8 and 16. For calves that shed oocysts on more than one visit, the clinical data of the visit when the highest excretion intensity was seen were included in the statistical analyses.

To achieve a sufficient amount of oocysts, positive samples were pooled by farm and suspended with water to achieve a fluid consistency. Oocysts were purified from these suspensions according to Joachim et al. (2003). In short, suspensions of faeces were passed through a series of sieves of decreasing mesh size, pelleted, resuspended in water and centrifuged with ether to remove lipid debris. After removing the ether by washing with water the sediment was resuspended in cold saturated sodium chloride (specific gravity 1.2) and the suspension was carefully overlaid with cold distilled water. The sample was then centrifuged (2300 *g*, 10 min, no brake) and oocysts were collected from the layer that formed between saturated sodium chloride and distilled water. The oocyst suspension was washed twice with distilled water (2300 *g*, 8 min) and stored at 4 °C in phosphate buffered saline (PBS) supplemented with penicillin/streptomycin and amphotericin B. Due to laboratory procedures the length of storage before use in cell culture varied between different field isolates (Table 1). *C. parvum* oocysts of all purified field isolates were subtyped by *gp60* PCR as reported before (Holzhausen et al., 2019).

Immediately prior to *in vitro* exposure of HCT-8 monolayers to parasite invasion, oocysts of all isolates including the reference strain were incubated for decontamination in 4% sodium hypochlorite on ice for 5 min and then washed twice with 1 ml sterile PBS. The resulting oocyst pellet was resuspended in growth medium 1 (RPMI (Gibco ™, Grand Island, USA), 1% sodium pyruvate, 5% foetal calf serum (FCS)) and thereafter oocysts concentration densities were estimated by counting oocysts in a Neubauer chamber. HCT-8 cells were seeded into 96 well cell culture microplates (7×10^4/well) and grown for 24 h (37 °C, 5% CO_2) in growth medium 2 (RPMI, 1% sodium pyruvate, 10% FCS) to confluent monolayers (confluence > 90%). Oocysts of each farm isolate were inoculated into separate HCT-8 monolayers. To facilitate infection of monolayers growth medium 2 was replaced by excystation medium (growth medium 1 supplemented with 0.4% sodium taurocholate) when oocysts were added. Penicillin/streptomycin and amphotericin B were added to every well to prevent growth of other microorganisms. After 4 h of excystation the medium was again replaced by growth medium 1 or PBS (PC, see below), respectively. Incubation (37 °C, 5% CO_2) was continued for 24 h. Thereafter growth medium 1 was replaced by RPMI without any additives. 10 µl of MTT-solution (0.5% Thiazolyl blue, Carl Roth GmbH, Karlsruhe, Germany) was added to every well and incubation was continued for another 4 h, followed by addition of 100 µl of 10% SDS in 10 mM HCl to each well to stop further cleavage of MTT. The microplates were then incubated at 37 °C and 5% CO_2 overnight before OD values were recorded at 595 nm for each well (ELISA-Reader Anthos 2000, Anthos Labtec Instruments GmbH, Wals-Siezenheim, Austria). Every assay was performed in triplicate. Uninfected cells cultured in growth medium 1 served as negative control (NC) and cells cultured in PBS after removing of excystation medium served as positive control (PC). Three cell-free wells contained RPMI only to determine blank values (blank control, BC).

Table 1
Length of storage at 4 °C of *C. parvum* field isolates after purification of oocysts and before evaluation of cytopathogenicity in HCT-8 cell monolayer.

isolate ID	storage time (weeks)
4	26
5	26
7	26
12	16
13	13
14	13
16	14
18	14
19	11
24	8
34	12
35	12
36	12
38	12
41	9
43	3
44	3
46	< 1
47	< 1
48	< 1
49	4
50	4
53	1
54	1
55	1
56	2

Cell viability of each infected well was calculated based on OD as follows:

$$\text{cell viability}\,(\%) = \frac{\text{OD isolate - OD BC}}{\text{OD NC - OD BC}}$$

OD NC: mean of OD value of triplicate NC
OD BC: mean of OD value of triplicate BC

Before evaluating cytopathogenicity of the field isolates, a preliminary test was carried out with different infection doses of the reference strain (6.25×10^4, 1.25×10^5, 2.5×10^5, 5×10^5 and 1×10^6 oocysts per well) and uninfected controls. During microscopic control of the infected cells a confluent monolayer was still visible in wells infected with 6.25×10^4, 1.25×10^5 and 2.5 $10x^5$ oocysts per well. In monolayers infected with 5×10^5 or 1×10^6 oocysts per well cell destruction was obvious (Fig. 1) and apparently on a similar level (student's *t*-test of OD values: $p > 0.05$). Therefore, the infection dose of 5×10^5 oocysts per well was selected for the evaluation of cytopathogenicity of the field isolates. According to the calculated cell viability (%) values, three categories of cytopathogenicity were defined for the subsequent MTT assays as follows: > 80% cell viability (no or low cytopathogenicity) = category 1; 40% - 80% cell viability (moderate cytopathogenicity) = category 2; < 40% cell viability (high cytopathogenicity) = category 3.

Descriptive statistics for cell viability values and the clinical data were performed using Microsoft Excel 2010 (Micosoft Cooperations, Remond, USA). The interquartile range (IQR) is defined as the data between the first and third quartiles. Evaluation of data for normal distribution (Shapiro-Wilk-test) and correlation analyses (Pearson's correlation coefficient, r) were performed using SPSS statistics version 25® (IBM, Armonk, USA).

3. Results

Cell viability was assessed for field isolates pooled per farm in comparison to non-infected control monolayers (NC = 100%) at a standard infection dose of 5×10^5 oocysts. In all, cytopathogenicity of the tested field isolates varied considerably with cell viability values of 17.7% (± 5.1%) to 99.5% (± 7.1%) (Fig. 2). Nine field isolates did not distinctly affect cell viability and were graded into category 1 (median host cell viability 93.8%; IQR 86.7 % - 98.2%). Cells infected with the reference strain showed cell viability of 63.0% (± 18.6%) in comparison to NC (category 2). Thirteen field isolates reduced cell viability to 62.7% (IQR 54.5% - 67.4%) and were also allocated to category 2. Cells infected with four different field isolates of category 3 showed a viability of 33.0% (IQR 25.7–37.9 %).

A significant positive correlation ($r = 0.60$, $p = 0.001$) was observed between the cell viability values after infection with different *C. parvum* field isolates and their storage time (in weeks). The median OCS of all calves tested positive for *C. parvum* on one farm correlated

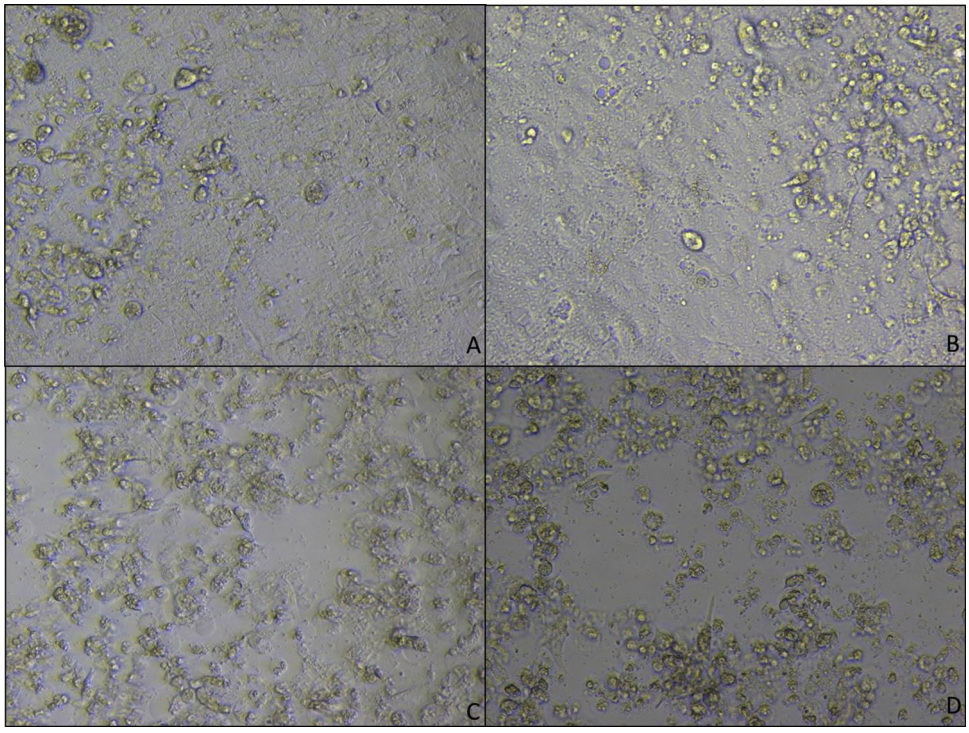

Fig. 1. HCT-8 cell layers 24 h post infection inoculated with *C. parvum* oocysts in different infection doses (in house reference strain, *gp60* subtype IIaA15G2R1). A: 1.25×10^5 oocysts per well; B: 2.5 $10x^5$ oocysts per well; C: 5×10^5 oocysts per well D: 1×10^6 oocysts per well.

significantly with the cell viability value of the respective field isolate in a negative manner ($r = -0.44$, $p = 0.026$). Because storage time of the oocysts was significantly correlated with their cell viability value, the relationship between these two parameters were additionally analyzed for isolates stored ≤ 4 weeks, ≤ 8 weeks, ≤ 12 weeks and ≤ 16 weeks. In all batches the median OCS gathered during the farm visits correlated negatively with the cell viability data with values of $r = -0.34$, -0.35, -0.34 and -0.32, respectively. However, the correlation was not significant ($p > 0.05$).

4. Discussion

Cryptosporidium is an apicomplexan parasite of public health concern and veterinary importance (Bouzid et al., 2013). Comparisons of virulence or infectivity between different strains or isolates of the parasite were reported in a mouse model (Sayed et al., 2016), in a human volunteer study (Okhuysen et al., 1999) and in calves infected with human or bovine isolates (Pozio et al., 1992).

In vitro assays based on infection of monolayers with *C. parvum*

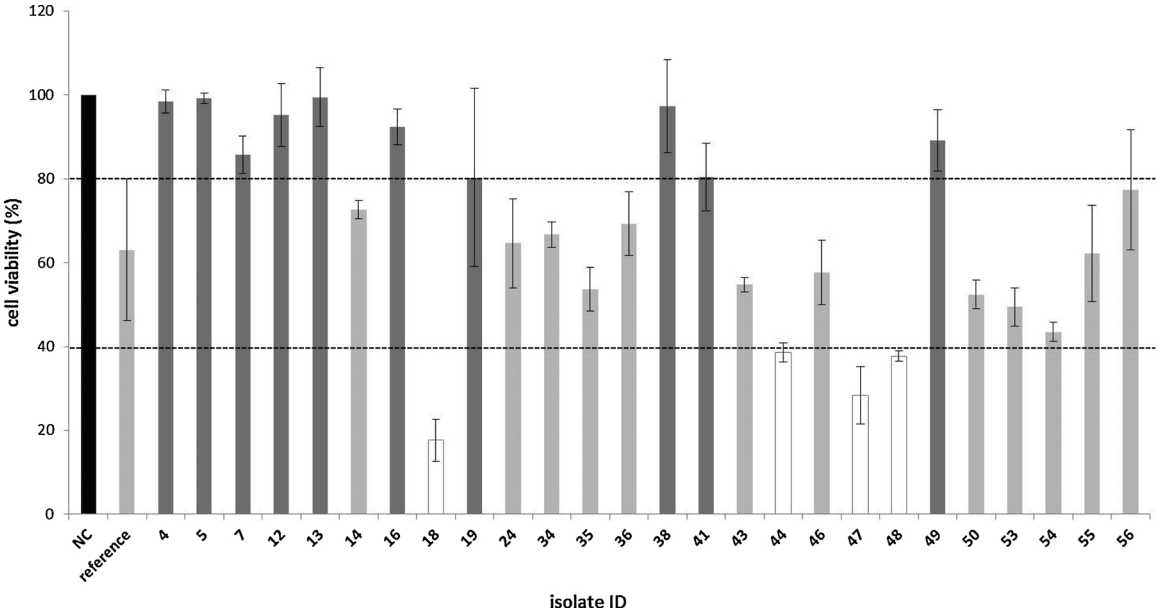

Fig. 2. Cell viability assessed in HCT-8 monolayers (7×10^4cells/well) after inoculation by 5×10^5 oocysts of *C. parvum* field isolates (n = 26). Monolayer cell viability was evaluated by MTT assay in comparison to uninfected cells cultured in the same well plate (NC). Dark grey = category 1: 3 > 80% cell viability (no or low cytopathogenicity); Grey = category 2: 40% - 80% cell viability (moderate cytopathogenicity); White = category 3: < 40% cell viability (high cytopathogenicity). Values are the medians ± standard deviation of three replicates.

oocysts have been successfully applied before to evaluate infectivity (Shahiduzzaman et al., 2010; Joachim et al., 2003; Delling et al., 2016). To the best of our knowledge *in vitro* comparison of cytopathogenicity of *C. parvum* field isolates were not published before. In the current study it was demonstrated that *C. parvum* field isolates in fact differ in their ability to alter host cell monolayers. MTT is a well-established method used in many laboratories to assess viability of cell monolayers, and we found that it is also suited to assess cytopathogenicity *in vitro* and to deliver data allowing the differentiation of field isolates according to this feature. Isolates that multiply in different rapidity may differ in cytopathogenicity if only a single period (in this study 24 h) of parasite inoculation is considered and the cytopathogenic effects of second merogony or gamogony cannot be determined (Hijjawi et al., 2001). On the other hand, Mele et al. (2004) reported that early apoptosis is induced already when *C. parvum* sporozoites bind to the host cell membrane and Delling et al. (2017) did not detected a significant difference of *C. parvum* DNA copies between incubation periods of 24 h or 48 h. However, further validation by e. g. quantitative real - time PCR over a longer period of incubation is needed to ensure that differences in the MTT assay are not only related to the replication speed of farm isolates.

Assessment of *C. parvum* virulence is difficult if not impossible in the field due to the many factors that may obscure the contribution of *C. parvum* to the clinical picture, which is basically represented by diarrhoea and related health impairment and thus rather unspecific. Data acquired in the laboratory does not fully reflect the natural situation and needs to be interpreted in the context of clinical and epidemiological features of the pathogen. However, the current study shows a relationship between the estimated viability of HCT-8 cell monolayers after infection with *C. parvum* and the clinical status of the calves infected with the respective isolate. According to Rochelle et al. (2002) *in vitro* infectivity may vary due to general assay variation, nonetheless these authors reported that estimation of infectivity of *C. parvum* in cell culture and mouse assays are comparable. In the current study a standard deviation of 18.6% in cell viability between replicates performed for the in house reference strain was observed. Since variation is intrinsic to assays that are based on infection of cell cultures we propose that grouping of values into categories of cytopathogenicity as done in this study is justified and more meaningful than to assume a precision of values. In accordance with Jenkins et al. (2003), the storage time of the oocysts and cell viability values correlated significantly with a higher amount of cell survival after infection with isolates stored for a longer time period. These findings were considered by evaluation of cell viability values regarding their correlation to the clinical data of the calves infected with *C. parvum*. Analyses of isolates, which were stored longer than 4, 8, 14 or 16 weeks, did not result in any significant correlation. To exclude the other factors that influenced the occurrence of diarrhoea in the calves and to attribute the symptoms only to cryptosporidiosis, a well-defined animal infection model would help to clarify the relationship between cell viability data and the clinical course of the infection. However, this requires extensive animal experimentation in calves which raises significant ethical concerns. Nonetheless our data may stimulate further experimental investigation into phenotypic features of *C. parvum* isolates.

5. Conclusion

To conclude, we propose our assay as a tool to screen *C. parvum* isolates for *in vitro* cytopathogenicity. It may be suitable to characterize phenotype variability related to virulence and thus help to better understand the different appearance of the disease in the field.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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