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RESEARCH ARTICLE

Could Vital Dyes be used to Determine the Degree of the Time Dependent Viability Changes in *Cryptosporidium parvum* Oocysts?

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Summary

The present study was undertaken to determine time dependent viability changes of purified *Cryptosporidium parvum* oocysts, stored in antimicrobial-supplemented PBS at +4°C, using vital dyes (DAPI/PI). The trials demonstrated that vital dyes could provide estimation of oocyst viability, and furthermore, if interpreted correctly, they could be used to determine the degree of the viability in Cryptosporidium parvum oocysts as cell culture-PCR assay is used.

Keywords: *Cryptosporidium parvum, Viability, Vital dye*

Cryptosporidium parvum Oocystlerindeki Zamana Bağlı Canlılık Değişim Derecesi Vital Boyalarla Belirlenebilir mi?

Özet

Bu çalışmada, antimikrobiyal destekli PBS içerisinde +4°C'de muhafaza edilen pürifiye *Cryptosporidium parvum* oocystlerindeki canlılık değişimi vital boyalarla (DAPI/PI) belirlenmeye çalışılmıştır. Denemeler, vital boyaların oocyst canlılığındaki değişimi ortaya koyabildiğini ve ayrıca iyi değerlendirildiği taktirde, canlılıktaki söz konusu değişimin derecesinin de, hücre kültürü-PCR uygulamalarına benzer şekilde belirlenebileceğini göstermiştir.

Anahtar sözcükler: *Cryptosporidium parvum, Viabilite, Vital boya*

INTRODUCTION

Determination of *C. parvum* oocyst viability requires attention in several regards, such as determination of the effectiveness of disinfection methods. Cell culture methods [1,2], animal experiments [3], vital dye stains [4], excystation methods [5] and the RT-PCR technique [6] are employable for the demonstration of viability changes in *Cryptosporidium* oocysts under the influence of various factors. Oocyst viability is stated to be determined correctly using the vital dyes DAPI-PI [4]. On the other hand, studies have pointed out the possibility of problems related to the efficiency of this technique [7-9].

The present study was carried out to investigate time-dependent viability changes of purified *Cryptosporidium parvum* oocysts using the vital dyes 4',6-diamidino-2-phenylindole-dihydrochlorid (DAPI) and propidium iodide (PI). The effectiveness of this method is compared to the results gained from a cell culture polymerase chain reaction (PCR) assay used as a control method.

MATERIAL and METHODS

C. parvum Oocysts: In the present study, a German

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field isolate of *C. parvum* from cattle was used. Oocysts were passaged *in vivo* in calves 3, 9 and 12 months before use. The three obtained oocyst batches were stored at +4°C in phosphate buffered saline supplemented with penicillin, streptomycin, and amphotericin B, which was renewed monthly.

Cell Culture-PCR Assay: Cell culture PCR assay was used to prove the viability of the oocyst batches used in vital dye staining trials. For the maintenance of the permanent cell culture (human ileocaecal carcinoma HCT-8 cells; ECACC, European Collection of Cell Cultures, Cat. No. 90032006) and for the cell culture-PCR assay, the method described before [10] was used. For PCR assay the *C. parvum* specific primer set CP 3.4-3′ and CP 3.4-5′ was used which amplifies a region of 650 bp in length [11].

Staining of Oocysts with DAPI/PI: Viability changes in the oocysts examined were demonstrated using DAPI (AppliChem, Darmstadt, Germany) and PI (Sigma-Aldrich, Taufkirchen, Germany) as described by Campbell et al. (1992) [4]. In brief, working solutions of DAPI (2 mg/ml in absolute methanol) and PI (1 mg/ml in 0.1 M PBS, pH 7.2) were prepared and stored at +4°C in the dark. For each oocyst batch, a total of 10^5 oocysts were suspended in 100 µl of PBS, 10 µl of DAPI working solution and 10 µl of PI working solution were added and the resulting suspensions were vortexed briefly and then incubated at 37°C for 2 h. Following incubation, the samples were washed twice in PBS, and viewed under a fluorescence microscope (DM IRB, Leica, Bensheim, Germany) equipped with a UV filter block (350-nm excitation, 450-nm emission) for detection of DAPI fluorescence, and a green filter block (500-nm excitation, 630-nm emission) for PI fluorescence detection. For each sample, at least 100 oocysts were counted. Oocysts which fluoresced bright red under the green filter block were considered PI(+), while oocysts which fluoresced sky blue entirely or at the level of the nuclei of the sporozoites under the UV filter block were considered DAPI(+). Oocysts which showed an absence of fluorescence for both dyes and displayed a normal oocyst structure under interference contrast optics were considered DAPI(-)PI(-).

The data were analyzed using the Kruskal-Wallis test. P-values of less than 0.05 were considered as statistically significant.

RESULTS

Cell Culture-PCR Assay: 3-months old *C. parvum* oocysts were tested positive for *C. parvum* DNA when 10^6, 10^5, 10^4, 10^3 or 10^2 oocysts, respectively, were seeded per well. For both of 9- and 12-months old oocyst batches, positive PCR results were gained from wells containing 10^6, 10^5, 10^4 and 10^3 oocysts.

Staining of Oocysts with DAPI/PI: By vital staining, it was found that 3-, 9- and 12-months old oocysts featured a PI positivity rate of 7.6±0.2%, 48.5±0.3%, and 53.3±0.4%, respectively. Concurrently, the same examined oocysts showed DAPI positivity rates of 15.6±0.3%, 66.7±0.7%, and 75.3±0.9%, respectively *(Table 1)*.

With decreasing age of the examined oocyst batch the rate of DAPI(-)PI(-) *(Fig. 1. 3, 4)* increased. Some of them gave a ghost-like homogenous blue reflection under the UV filter block *(Fig. 1. 4)*. The oocysts which were DAPI(+) not in toto but in terms of a typical blue fluorescence of the nuclei generally were PI(-) *(Fig. 1. 2)*. Yet, our

Table 1. *Results of DAPI-PI staining in 3 (A), 9 (B) and 12 (C) month-old oocysts (X ±Sx)*

Tablo 1. *3 (A), 9 (B) ve 12 (C) aylık oocystlerde DAPI-PI boyama sonuçları (X ±Sx)*

Oocyst Batch	Excysted Oocyst (% X ±Sx)	PI(+) (% X ±Sx)	DAPI(+) (% X ±Sx)
A	4.3±0.2^c	7.6±0.2^c	15.6±0.3^c
B	6.0±0.3^b	48.5±0.3^b	66.7±0.7^b
C	14.0±0.3^a	53.3±0.4^a	75.3±0.9^a

^{abc} *For each batch (regarding a single column), different letters indicate statistically significant differences (P<0.05)*

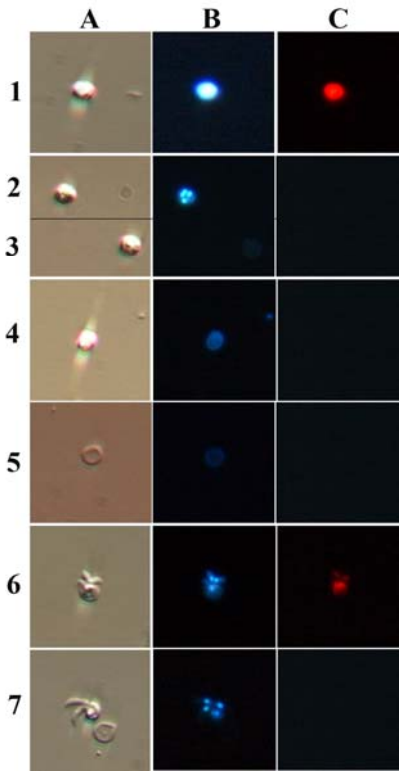

Fig 1. Direct interference contrast, UV filter block (DAPI) and green filter block (PI) images of oocysts following the application of DAPI/PI staining (x400 magnification)

Şekil 1. DAPI/PI uygulamasını takiben oocystlerin direct interferens contrast, UV filtre (DAPI) ve yeşil filter (PI) düzenekli mikroskop görüntüleri (x400 büyütme)

observation showed that DAPI(+) oocysts with an overall diffuse blue fluorescence usually were PI(+) at the same time *(Fig. 1. 1)*. All PI(+) oocysts were also DAPI(+) *(Fig. 1. 1, 6)*. The excysted and empty oocysts excysted were generally viewed as light bluish ghosts *(Fig. 1. 5)*. All of the at least partially excysted sporozoites were determined to be DAPI(+) *(Fig. 1. 6, 7)*, whereas some were PI(+) *(Fig. 1. 6)* and some other were PI(-) *(Fig. 1. 7)*.

DISCUSSION

Our data show that under the given experimental conditions, the viability of *C. parvum* oocysts decreased with time, though viability was maintained to a certain degree for more than 12 months as confirmed by previous studies [12,13]. These time-dependent changes were demonstrated successfully by cell culture PCR assay. However, viability differences between 9 and 12 months old oocysts could not be displayed clearly by conventional PCR. Thus, quantitative PCR designed for this purpose [14,15] is needed to exhibit viability differences between oocyst batches with similar viability levels.

Concerning DAPI and PI, which are specific nuclear dyes, it is reported that DAPI may pass through the walls of intact oocysts and sporozoites, whereas PI does not, and therefore viable oocysts are stained DAPI(+)PI(-). It was reported that the permeability of the oocyst wall is minimal directly after faecal oocyst excretion. However, also some of the older oocysts are stated to take up neither of both dyes. Wall permeability may be increased by acid pre-treatment [4,16], exposure to heat [17], several chemical treatments [7,18,19], or intermediate UV exposure [8,9]. After the artificial permeabilization the oocysts also render DAPI(+) and may excyst. Consequently, DAPI/PI staining gives rise to much higher viability levels after such applications.

In the present study, DAPI(+) and PI(+) levels in oocysts were determined to increase directly proportionally to the length of time when they were maintained at +4°C. As also reported previously [17], it was seen that oocysts which lost their viability to a large extent are both DAPI(+) and PI(+). Due to the damage of the nuclear structure of sporozoites and the disintegration of the nuclear content, oocysts of this kind did not display typical nuclear staining and were observed to give homogenously distributed blue fluorescence with DAPI staining. Our findings revealed that the main barrier for DAPI was the intact oocyst wall, and the intact sporozoite membrane was an important barrier for PI. This result could be understood best from partially excysted sporozoites.

For the oocyst batches examined, results of the PI staining came out as expected and were similar to the results of the viability assay using cell culture; yet, conflicting results were obtained for DAPI which revealed higher viability in older oocysts. Nevertheless, overall comparative data obtained in the study suggest that DAPI/PI staining, if interpreted correctly, may be used effectively for the determination of time-dependent changes in the viability.

In conclusion, it is obvious from our results that 1) PI staining, similar to cell culture, determines time-dependent viability changes. 2) Oocysts which possess high viability rates stain DAPI(-)PI(-). 3) If an oocyst is DAPI(+)PI(-), it may be vital, yet it rapidly loses its viability, and should be passaged immediately.

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