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RESEARCH ARTICLE

Determination of Developmental Stages of *Cryptosporidium parvum* in HCT-8 Cell Culture by Differential Interference Contrast Microscopy, Giemsa and Haematoxylin-Eosin Staining Methods

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Summary

This study aimed to compare the advantages of differential interference contrast microscopy, Giemsa and haematoxylin-eosin staining techniques for detecting, visualizing and distinguishing the developmental stages of *Cryptosporidium parvum* in cell culture. Data showed that interference contrast microscopy and Giemsa staining have certain advantages, whereas haematoxylin-eosin staining followed by formalin fixation clearly reveals certain changes occurred in the parasite. In the investigations, it was revealed that direct microscopy is effective in accurate definition of meronts and microgamets, but staining methods can differentiate meronts only.

Keywords: *Cryptosporidium, Cell culture, Giemsa, Haematoxylin-Eosin*

Cryptosporidium parvum'un HCT-8 Hücre Kültüründeki Gelişme Evrelerinin Diferansiyel İnterferans Kontrast Mikroskopisi, Giemsa ve Hematoksilin-Eozin Boyama Yöntemleriyle Belirlenmesi

Özet

Bu çalışmada, interferans kontrast mikrsokopisi, Giemsa ve hematoksilin-eosin boyama yöntemlerinin, *Cryptosporidium parvum*'un hücre kültüründeki gelişme evrelerinin belirlenmesi konusundaki yetilerinin karşılaştırılması amaçlanmıştır. Elde edilen veriler, interferans kontrast mikroskopisi ve Giemsa boyama yönteminin söz konusu parazitin gelişme evrelerinin belirlenmesinde bazı önemli avantajlara sahip olduğunu, formalin ile tespit işlemini takiben uygulanan hematoksilin-eosin boyama tekniğinin ise parazitte meydana gelen bazı değişikliklerin kolayca belirlenmesini sağladığını göstermiştir. Taramalarda, interferans kontrast mikroskopisi ile meront ve mikrogametlerin kesin olarak ayırt edilebileceği, boyama yöntemleriyle ise sadece merontların belirlenebileceği anlaşılmıştır.

Anahtar sözcükler: *Cryptosporidium, Hücre kültürü, Giemsa, Hematoksilin-Eosin*

INTRODUCTION

Cell culture can be used for the study of biology, infectious capability, vitality level of *C. parvum,* in addition to the effect of different chemicals on this parasite [1-3]. Although different cell culture systems have been devised for the studies with *C. parvum*, HCT-8 (Human Colonic Tumor Cells) can be used successfully, since this culture could be maintained as much as 25 days by means of the passages at 2-3 days intervals [4]. However, there are some disadvantages of producing the parasite in cell culture. First of all, parasite cannot complete its whole life cycle in cell culture [5]; in case of completion, the number of reproduced oocysts is lower than the inoculated oocysts. Furthermore, the cell culture designed for this parasite cannot continue indefinitely [6,7]. In the studies carried out with different cell cultures, it was reported that the first forms of the parasite could be seen in 90th min of the culture [8], while trophozoites, type I and type II meronts, macrogamets, microgamets, and immature and mature

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oocysts [7,9] within 72 h. Yet, the auto re-infective activity of the parasite starts at 12th h [10], trophozoites and meronts form at 24th h, all other forms could be observed between 48th and 72nd h [11], both immature and mature oocysts could be seen at 72nd h [4] when the parasite is cultured in HCT-8 cells.

The aims of the present study are to detect and describe the developmental stages of *C. parvum* in cell culture, and to evaluate the success of direct interference contrast microscopy, haematoxylin-eosin and Giemsa staining methods in the examination of this parasite in culture.

MATERIAL and METHODS

Oocyst Preparation

A calf genotype of *C. parvum* was used in the study. Oocysts were obtained from the faeces of an experimentally infected calf. They were stored at 4°C in phosphate buffered saline (PBS) containing penicilline (100 U/ml), streptomycine (0.1 mg/ml) and amphotericin B (2.5 µg/ml) until use for a period of 4 months. PBD was changed monthly.

In vitro Infection to the Cell Line

HCT-8 cell line (ECACC, European Collection of Cell Cultures, and Cat. No. 90032006) was used as the permanent cell culture. HCT-8 cells were maintained in 24-well cell culture plates [2]. Just before inoculation, round cover slips of 8 mm in diameter were placed to the floor of each well. The cover slips were firstly flame-sterilized and then cooled in air to avoid sticking to the base of the plate. $2x10^5$ cells were inoculated to each well and incubated in a 5% CO_2 atmosphere at 37°C. Subsequently, 10^4 oocysts were inoculated to each well that was grown to 80-90% cell confluence on cover slips [3].

Interference Contrast Microscopy

At the 24th, 48th and 72nd h of the incubation, the wells were examined with an inverted microscope equipped with Nomarski interference-contrast optics, the required images were recorded, and the developmental stages of the parasite detected in different 20 microscopic fields were counted for each well.

Subsequently, medium in the wells was removed, and the cells were washed with Dulbecco's PBS. Each cover slip taken from wells was subjected to staining methods.

Giemsa Staining

Cells on cover slips allocated for Giemsa staining were fixed with cold absolute methanol for 10 min. They were then air dried, and stained with 5% Giemsa solution for 25-30 min. After washing slightly under tap water, they were left to dry, and mounted. Afterwards slides were examined under a light microscope at x100 magnification. The relevant cells were photographed, and the developmental stages of the parasite detected in different 20 microscopic fields were counted from each slide.

Haematoxylin-Eosin Staining After Methanol Fixation

Following fixation as in Giemsa staining, cover slips were kept in a haematoxylin solution for 5 min and then washed with distilled water gently. They were rinsed under running tap water for 10 min. After washing again with distilled water, they were left into eosin solution for 5 min, and then washed with distilled water. The cover slips were passed through 96% ethanol twice for 2 min, and left to air dry. The remaining procedure was the same as Giemsa staining.

Haematoxylin-Eosin Staining After Formalin Fixation

The same procedure was followed, with the exception that cells were fixed with 10% formalin for 30 min.

Identification of the Foci

The description of the forms of the parasite was based on the studies by Hijjawi et al.[4], Lacharme et al.[7], Mele et al.[11], Villacorta et al.[12], Woodmansee and Pohlenz [13], Yu et al.[14], Flanigan et al.[15], Lawton et al.[16].

RESULTS

The number of the parasite forms was expressed as field average (minimum-maximum). With the examination under interference contrast microscope at x40 magnification, the count was found 36.5 (8-50) at 24th h, 51.3 (9-62) at 48th h and 30.0 (4-39) at 72nd h. However, Giemsa stained slides examined at x100 exhibited parasite numbers 27.4 (4-40), 18.2 (0-30) and 7.3 (0-18) at 24th, 48th and 72nd h, respectively. The results for the slides methanol fixed and haematoxylin-eosin stained were 22.7 (0-30) at 24th h, 11.5 (0-17) and at the 72nd h 3.8 (0-9), while the counts for the slides fixed with formalin and stained with haematoxylin-eosin were 21.5 (0-29) at 24th h, 6.2 (0-12) at 48th h and 0.4 (0-5) at 72nd h. All the results were summarized in *Table 1*.

Interference contrast microscopy displayed that the forms were placed side by side in single or multiple groups, and meronts at the 48th h of the incubation and microgamets at the 72nd h were defined. On the other hand, only meronts could be described at the 48th h in Giemsa stained slides *(Fig. 1)*.

In the slides stained with haematoxylin-eosin after formalin fixation, the cytoplasm of the HCT-8 cells at the

24th h was more homogenous. At the 48th h, the nuclei of these cells darkened and became more pronounced, the cytoplasm was vacuolized. At the 72nd h, darkness of the nuclei was higher, and vacuolization of the cytoplasm was increased.

the stained slides by taking distinctive criteria in the current literature into consideration, many forms could not be defined absolutely according to those criteria. Interference contrast microscopy, haematoxylin-eosin and Giemsa staining techniques were reported to be

Table 1. *Count of the forms of Cryptosporidium parvum developing in HCT-8 cell culture at different times, monitored by different techniques [field average (min-max)]*

Tablo 1. *Cryptosporidium parvum'un HCT-8 hücre kültüründe, değişik zamanlarda gelişen ve farklı yöntemlerle belirlenmiş olan form sayıları [saha ortalaması (min-mak)]*

Method	Count of the Parasite Forms at Different Times of the Culture		
	24th Hour	48th Hour	72nd Hour
Interference contrast microscopy	36.5 (8-50)	51.3 (9-62)	30.0 (4-39)
Giemsa staining	27.4 (4-40)	18.2 (0-30)	7.3 (0-18)
Haematoxylin-eosin staining (after methanol fixation)	22.7 (0-30)	11.5 (0-17)	3.8 (0-9)
Haematoxylin-eosin staining (after formalin fixation)	21.5 (0-29)	6.2 (0-12)	0.4 (0-5)

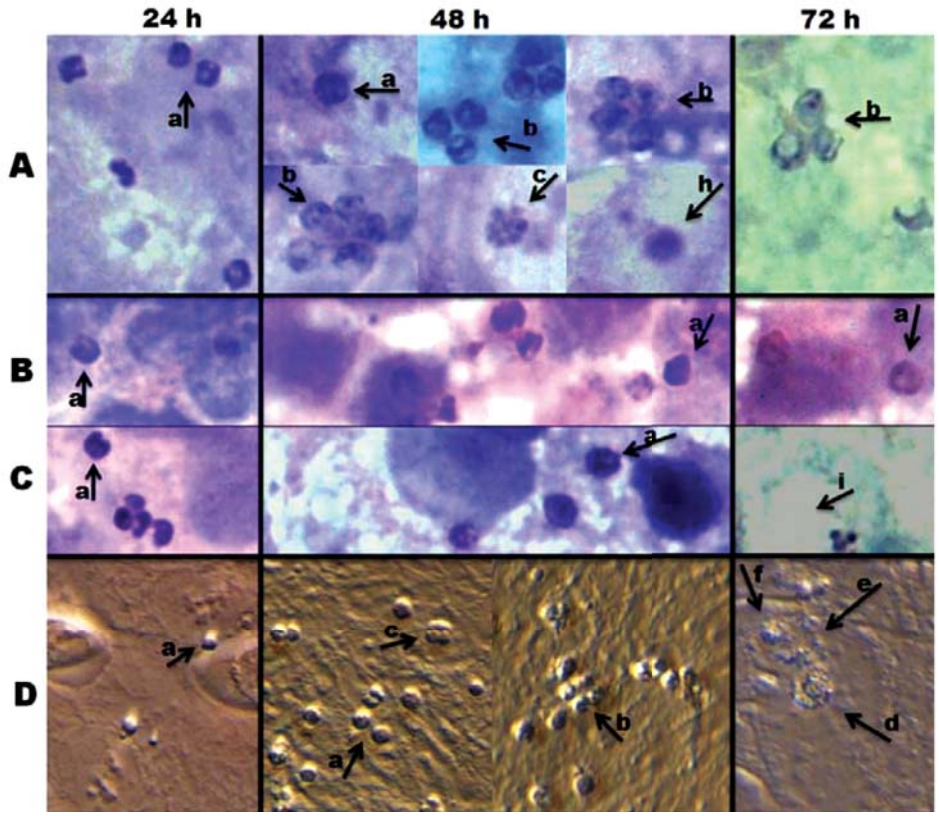

Fig 1. The developing forms of *Cryptosporidium parvum* in HCT-8 cell culture after Giemsa staining (x100) (A), haematoxylin - eosin staining (x100) (B: after methanol fixation, C: after formalin fixation), and in the differential interference contrast microscopy (x40) (D). a: single form, b: multiple form, c: meront, d: microgamont, e: microgametocytes directed to the macrogamet, f: macrogamet surrounded by three microgametocytes, h: HCT-8 cell inclusion, i: cell vacuolization

Şekil 1. *Cryptosporidium parvum*'un HCT-8 hücre kültüründe gelişen formlarının Giemsa boyama (x100) (A), haematoxylin-eosin boyama (x100) (B: Metanol tespiti sonrası, C: Formalin tespiti sonrası), interferans kontrast mikroskop görüntüleri (x40) (D). a: Tekli form, b: Çoklu form, c: Meront, d: Mikrogamont, e: Makrogamete yönelmiş mikrogametositler, f: Üç mikrogametosit tarafından kuşatılmış makrogamet, h: HCT-8 hücre inklüzyonu, i: Sitoplazma vakuolleşmeleri

DISCUSSION

The forms of *C. parvum* developing in cell cultures were reported to change according to the origin of the parasite, host cells and some culture conditions. The parasite could complete almost all developmental stages within first 72 h. The forms have their own specific size and structure, and they can be defined by using these characteristics [4,11]. Although we described the forms in successful in order to distinguish the forms in the culture [4,7,12], however, it was stated that identification is so difficult in the stained slides, and only meronts can be differentiated easily, depending on the sitoplasmic collapse occurring during the preparation [13].

In the studies with different cell culture systems, although the meronts were stated to be seen at the 24th [16], 48th [9,11] and 72nd [7] h, in the present study, typical meronts were particularly observed at the 48th h, and no

exact meront definition was made at the 24th and 72nd h. 4-8 mononucleate forms developing within a single cell and meronts resembled each other, but the meront nucleus was smaller than the multiple forms. On the other hand, in the interference contrast microscope, particularly 6-8 nucleate meronts possessed the nuclei being not side to side unlike to multiple forms, and this three-dimensional view of the meronts was useful for differentiating these two forms. However, these differences were not observed in the stained slides, because meront nuclei lost their three-dimensional view and had an appearance of flower petal as in the some 6-8 nucleate multiple forms. But here the size of nuclei was useful for differentiation.

It was stated that the development of the parasite could not pass beyond the asexual stage in cell culture [13]. Although both sexual and asexual development could occur, the oocyst could not form [5], or only immature oocyst could develop [16]. However, some studies revealed that oocysts with thick walls could develop at the 72nd h, but the thin walled type did not form [9,17]. Even though a few thin walled oocysts could form, they did not have the ability to leave the cell [18]. Although highly effective oocyst formation occurs in the culture [4], the number of oocysts is low [6]. The techniques and employed periods of time in this study did not allow an exact oocyst description. But, the forms which lost their smooth contour, had not a detailed interior structure, and possessed only a small, purple inclusion which is likely the residual body were thought to be oocysts in Giemsa stained slides prepared from the 72nd h of the culture. This was confirmed by the observations of the oocysts which were obtained from feaces and stained with Giemsa. However, the oocyst description based on the employed techniques needs additional confirmation.

The microgamonts were the forms which could be exactly defined in our study. They appeared as structures containing a big round core surrounded by small, round microgametocytes arranged in the interference contrast microcope. Such a definition was also made by Current and Long [18] for *C. parvum* cultured in the chicken embryo chorioallantoic membrane. These forms which were seen only at the 72nd h in the interference contrast microscope were not encountered in the stained slides. The cause of this may be the elimination of the forms during the staining process, since microgametocytes tend to leave the microgamete in their normal biological process.

Upton et al.[19,20] stated that the number of oocysts developed in the culture is lower than the number of inoculated oocysts, and this could be resulted from microgametocytes unable to leave the microgamont and to reach the macrogamete. However, our observations at interference contrast microscope showed that the microgametocytes move towards the macrogamete nearby with vibration-like motions. This observation made us think that this directed movement could be driven by some factors originating from the macrogamete. At this point, increasing the number of sexual forms by increasing the number of oocysts inoculated seems to be a solution in an attempt to elevate the number of oocysts developing in the culture. But, our studies demonstrated that increased number of oocysts inoculated gave rise to the host cell loss resulted from intensive sporozoite invasion. A study by Ojcius et al.[21] exhibited that the parasite causes apoptotic cell death. In our examinations, some changes including vacuolization and darkened nucleus were seen in the host cells.

In some studies conducted to determine the number of forms developed in *Cryptosporidium* cultures, direct microscopy or different staining methods were used, the number of forms developing in the culture was found to increase regularly within first 48 h, reaching a maximum at the 48th h [10], afterwards declining [8] and generally decreasing to the levels of 24th h at the 72nd h [7]. *C. parvum* cell culture in HCT-8 indicated that 82% of the cells were infected at the 24th h and 73% at the 48th h and 53% at the 72nd h [11]. In our observations of interference contrast microscopy, the number of forms developing in the culture was at the highest level at the 48th h, the density at the 24th h was relatively lower, and at the 72nd h the values in question were the lowest, being close to the 24th h. However, the average number of the forms obtained from the staining methods was the highest at the 24th h, decreased by time and reached the lowest value at the 72nd h. This decrease was seen especially in the slides stained after formalin fixation. This finding may be related to the less resistancy of the forms to fixation and staining protocols.

Our results clearly reveal that interference contrast microscopy and Giemsa staining have certain advantages in the determination of *C. parvum* forms developing in HCT-8 cell culture, and haematoxylin-eosin staining after formalin fixation is more appropriate in the determination of some changes caused by the parasite in the host cells. It was also understood that meronts and microgamonts in culture could be defined clearly with interference contrast microscope, and only meronts could be differentiated clearly by Giemsa staining.

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