Observation of Waterborne Protozoan Oocysts Using a Low-Vacuum SEM

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Cryptosporidium parvum (C. parvum), Cryptosporidium muris (C. muris), Cyclospora cayetanensis (Cyclospora) and Isospora belli (Isospora) are well known as the pathogenic protozoa of the waterborne-outbreak of diarrhea. Three species of protozoan oocysts infect and amplify into the epithelial cells of the small intestine and only the C. muris oocyst infects and amplifies into the gastric mucosal epithelial cells. C. parvum and C. muris oocysts are spherical with diameters of 4-10 µm. Cyclospora oocysts are spherical with diameters of 8-10 µm. On the other hand Isospora oocysts are elliptical 20-33 µm long and 10-19 µm wide. It is especially difficult and important to observe the microstructures, such as sporozoites, of C. parvum and C. muris with an optical microscope, because of a very robust oocyst wall that protects the four sporozoites in the environment for a very long time without their losing infectivity.

In this study, the shapes of oocyst walls and inner microstructures, such as sporozoites, were clearly observed with the low-vacuum scanning electron microscope (LV-SEM) without coating and with the samples of protozoan oocysts, prepared using the simple freeze-drying method. As a result of observations with LV-SEM, the oocyst walls of four species of oocysts could be observed at a magnification >1,000× and their microstructures such as sporozoites were clearly visible at 10,000×. It was concluded that the LV-SEM using our method is useful for observation of these protozoan oocysts.

Introduction

Cryptosporidium parvum oocyst (C. parvum) was well known protozoan parasite that was first discovered in the gastric mucosa of a mouse in 1907 [1]. The pathogenicity of C. parvum against human was not known early time, but since infection to immunocompromised persons was reported in 1976 [2], it has been described the attention in Europe and America as a cause of serious diarrhea symptoms to immunocompromised persons. Now C. parvum is recognized as one of the most commonly identified intestinal pathogens even to parsons with healthy immune systems [3]. And the infection to healthy infant of Cryptosporidium muris oocyst (C. muris) was reported in Indonesia in 1996 [4]. In Japan, as the first case of infection with C. parvum, more than 400 persons were reported in Kanagawa Prefecture in 1993. Then as the second case, about 8,000 people with diarrhea by waterborne infection of C. parvum were reported in Saitama Prefecture in 1996 [5]. In genus Cryptosporidium, it has been observed four sporozoites in the oocyst. C. parvum is parasitized in the mucosal epithelial cells of the small intestine and C. muris is parasitized in the gastric mucosal epithelial cells.

Cyclospora cayetanensis oocyst (Cyclospora) is a protozoa that parasitizes in mucosal epithelial cells of the human small intestine and causes diarrheal symptoms. Organisms of the genus Cyclospora have an oocyst with two sporozoites, each of which contains two sporozoites. In 1996, the largest diffuse outbreak of cyclosporiasis ever reported, affecting about 1,500 persons in North America [6], was associated with eating fresh raspberries; trace-back data indicated that the source of the berries was Guatemala.

On the other hand, Isospora belli oocyst (Isospora) is a protozoa that parasitizes in the mucosal epithelial cells of the human small intestine. It causes acute diarrheal symptoms against immunocompromised persons, including those infected with HIV [7].

In this study, we observed these four types of protozoan oocysts though a low vacuum scanning electron microscope (LV-SEM). We then compared this method and microscopic method according to the guideline set forth by the Ministry of Health, Labour and Welfare [8].

Material and Method

Collection of protozoan oocysts

We isolated protozoan oocysts by the centrifugal floating method using sucrose solution (specific gravity, 1.2) from the stool of patients, infected with C. parvum, Cyclospora and Isospora. Three species of protozoan oocysts as specimens were used for observation after fixing them with 10 % neutral formalin aqueous solution. We also used a specimen isolated from the bovine stool infected with C. muris in the same way.

Observation of C. parvum, C. muris, Cyclospora and Isospora through optical microscope

The four samples each of C. parvum, C. muris, Cyclospora and Isospora were dispersed onto the slides and dried at 55 to 60°C. The slides of C. parvum and C. muris were observed under B excitation, after staining them with the FL-Crypto-a-Glo kit (Waterborne Inc.) that is monoclonal antibody, which have been labeled directly with FITC, specific to the oocyst wall of Cryptosporidium. Cyclospora and Isospora were observed directly through a fluorescence microscope, because their oocyst walls emit blue auto-fluorescences under UV excitation (330-380nm). We then switched to a differential interference microscope to observe their oocysts and took micrographs.

Method of observing protozoan oocysts through low-vacuum SEM

Observation was conducted according to the following procedure using the method of observing and identifying C. parvum that was earlier reported by J. Suzuki et al. [9].

(1) A concentrated specimen was filtered through the SEM’s special-purpose specimen stage with an average pore diameter of 0.6 µm (JEOL Datum, Ltd.), depositing oocysts on the specimen stage of the SEM.

(2) 2 mL distilled water was dropped onto the specimen set on the specimen stage and the specimen was cleaned twice while aspirating it with a syringe attached under the specimen stage.

(3) The specimen stage was mounted on a specimen holder and was placed in an insulating container made of styrofoam; then liquid nitrogen was slowly poured around the specimen stage to freeze the specimen. The cooling time was about 40 seconds after the specimen had been frozen.

(4) The frozen specimen was quickly mounted on the specimen stage of a low-vacuum microscope and set on the specimen stage of a low-vacuum electron microscope. The cooling time was about 15 seconds after the specimen had been frozen.
Results and Considerations

Results of observation through Fluorescence Microscope

Monoclonal antibody kits for C. muris are not commercially available. We then stained C. muris using the monoclonal antibody kit for FITC marker anti-C. parvum and obtained an image on which the oocyst wall emits light-green fluorescence under B excitation as does the small C. parvum (Fig. 1a, 2a). We also observed Cyclospora and Isospora through a fluorescence microscope under UV excitation and obtained the images on which the oocyst wall emits light-blue fluorescence, as shown in Figs. 3a and 4a.

We observed the same field of view using a differential interference microscope and observed the stereoscopic structure of the four types of protozoan oocysts at a magnification of 1,000×, and identified sporozoites clearly in C. muris (Fig. 2b), but could not obtain a clear image of the sporozoite in C. parvum (Fig. 1b). Two sporocysts formed in a relatively thick oocyst (Fig. 3b) and the oocyst wall and internal cystoblast of Isospora could be observed through an optical microscope (Fig. 4b).

Therefore, of the four types of morphological protozoan oocysts observed through an optical microscope, the oocyst wall and internal structure of C. muris, Cyclospora and Isospora could be observed, but it was difficult to identify the internal sporozoites in C. parvum.

Results of observation of protozoan oocysts through low-vacuum SEM

The existence of C. parvum and C. muris could be confirmed through a low-vacuum SEM at a magnification of 1,000× or more (Fig. 1c, 2c). At a magnification of 10,000×, we could observe and identify the internal and external fine structures of sporozoites and oocyst walls that are difficult to observe through an optical microscope or a conventional scanning electron microscope that requires metallization with coating. Unlike C. parvum, which is small in size, C. muris is large and elliptical, but we could observe long and narrow sporozoites.

The existence of Cyclospora could be confirmed at a magnification of 500× or more and the oocyst wall could be clearly observed at a magnification of 8,000× (Fig. 3c). The internal structure of Cyclospora could be identified even by increasing the accelerating voltage to 25 kV and decreasing the pressure in the specimen chamber to 30 Pa, probably because the oocyst wall of Cyclospora is thick. However, observation for an extended time revealed that the specimen tends to become charged, and so, the observation conditions need to be corrected.

The existence of Isospora could easily be confirmed at a magnification of 500× or more, while oocyst wall and the internal structure SEM (JEOL Model JSM-5600LV), the specimen chamber was evacuated to 60 Pa, and the ice of the specimen in the LV-SEM was slowly and completely sublimated for about 60 minutes.

(5) After sublimating the specimen ice in the LV-SEM completely, the specimen chamber was evacuated to a pressure of 20 to 30 Pa and the accelerating voltage was set at 10 to 25 kV, and C. parvum, C. muris, Cyclospora and Isospora were searched for within a magnification range of 500× to 1,000×. The overall image and the internal structure of each oocyst were observed within a range of 5,000× to 10,000×.
sporoblast could be observed at a magnification of $3,000\times$ (Fig. 4c).

Observation of four types of protozoa oocysts of *C. parvum*, *C. muris*, and *Isospora* through a low-vacuum SEM revealed that the LV-SEM is very useful in observing the minute internal structure of oocysts and that the size and morphology of oocysts and sporozoites can be compared and examined relatively easily using the images stored in a computer, especially for two types of *Cryptosporidium*. Furthermore, even the internal structure of *Cyclospora* with a thick oocyst wall could be observed by increasing the accelerating voltage.

**Conclusion**

Specimens of four types of protozoan oocysts: *C. parvum, C. muris, Cyclospora* and *Isospora* were prepared by the freeze-drying method without coating within a short time and were observed within a magnification range of $500\times$ to $10,000\times$. The results showed that even the internal structure of *Cyclospora* oocyst with a thick oocyst wall can be observed by increasing the accelerating voltage to 25 kV and decreasing the pressure in the specimen chamber to 30 Pa. On the other hand, a problem was found in that the specimen tends to become charged during prolonged observation. The walls and internal structures of *C. parvum, C. muris* and *Isospora* oocysts could be observed at the accelerating voltage of 10 kV to 15 kV. Thus we found that protozoa oocysts can be observed efficiently and effectively through a low-vacuum SEM using the freeze-drying method.

**References**