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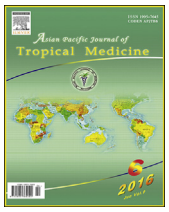

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Oyster is an effective transmission vehicle for *Cryptosporidium* infection in human

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ABSTRACT

Objective: To determine the ability of oysters to trap and maintain viable *Cryptosporidium* oocysts, and the feasibility of *Cryptosporidium* multiplication in oysters' organs.
Methods: Seventy oysters were raised in experimentally seeded natural seawater for up to 3 months, with weekly oocysts inoculations. *Cryptosporidium* oocysts, viable and non-viable, as well as other stages were detected using two immunofluorescence vital staining techniques (Sporo-Glo and Merifluor®) with confocal microscopy. Viability rate at various times after inoculations were calculated.
Results: *Cryptosporidium* oocysts were found most concentrated in oysters' digestive organs than in gill and water inside the oysters. Oocysts numbers were 857.33 at 24 h after inoculation and strikingly decreased to 243.00 and 126.67 oocysts at 72 h and 7 days, respectively. The oocysts in oyster were also less viable over time; 70%, 60% and 30% viable at 24 h, 72 h and 7 days after inoculation, respectively. At 77 days, the number of oocysts was very low and none was found at 84 days onwards. Although some oocysts were ruptured with released sporozoites, there was no evidence throughout the study of sporozoites multiplication to indicate that oyster is a biological host. Despite the significant reduction in oocysts number after 7 days of inoculation, the remained viable oocysts can still cause cryptosporidiosis.
Conclusion: The findings confirm that *Cryptosporidium parvum* does not multiply in oyster, and is therefore not a biological host. Nevertheless, the results suggest that oyster can be an effective transmission vehicle for *Cryptosporidium* oocysts, especially within 24–72 h of contamination, with viable oocysts present at up to 7 days post infection. Unless consuming well-cooked oyster dishes, eating raw oyster remains a public health concern and at least 3 days of depuration in clean sea water prior to consumption is recommended.

1. Introduction

Cryptosporidium is a coccidian protozoan parasite that infects various animals including humans. Zoonotic transmission of *Cryptosporidium parvum* (*C. parvum)* is a recognized public health concern [1,2]. Although an infected host often excreted large number of oocysts, relatively few are required to initiate the infection. In addition to being small, buoyant, the oocysts are resistant to harsh conditions even upon exposure to a wide range of temperature and salinities for weeks up to months [3]. Runoff water from urban or rural landscape, waste water outfall from household, untreated water from agriculture and farming are the causes of parasitic contamination to marine environment, affecting a variety of bivalves. Previous research demonstrated that *C. parvum* can multiply *in vitro* [4,5] and in biofilm [6]. Due to this capability of growing and spreading in extensive conditions, *C. parvum* is a highly adaptable pathogen and may not necessarily require specific biological host to grow.

Bivalves feed on suspended phytoplanktons, which are pumped up across the gill by ciliary action. Thus by non-

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selective filtration, pathogenic microorganisms may also be taken up and concentrated in the digestive glands [7]. Among the marine bivalves, oyster is often consumed raw, and 18 survey publications worldwide, excluding Thailand, have reported that the presence of *Cryptosporidium* oocysts contamination [8].

Whilst oyster acts as transmission vehicle, the present study was to determine whether the innoculated *Cryptosporidium* oocysts to be taken up, be viable, and persist within the oysters, and to determine the change viability and multiplication capacity over 3 months in seawater. Therefore, 70 oysters [*Crassostrea belcheri (C. belcheri)*], large size filter feeders found in Western and Southern regions of Thailand, were selected for this study.

2. Materials and methods

2.1. Source of C. parvum oocysts

The *C. parvum* cattle isolate (Swiss Cattle C26) was obtained from the School of Veterinary and Life Sciences, Murdoch University, and had been maintained in our laboratory. Briefly, 150000 oocysts were infected in 3-day old ICR mice for 8 days of amplification. Following euthanization, all parts of intestines were removed, minced, and oocysts were purified using ether extraction and Ficoll density gradient as previously described [9]. Purified oocysts were stored in phosphate buffered saline (PBS) and antibiotics (10000 U penicillin G, 00.1 g/L streptomycin) at 4 °C prior to use. The methods for animal use were approved by the Faculty of Tropical Medicine – Animal Care and Use Committee (Reference: FTM-ACUC 008/2013).

2.2. Experiment 1: detection and viability study of Cryptosporidium

A total of 18 similar commercial-size oysters (*C. belcheri*) were randomly selected for experiment 1, which was conducted at Samutsongkhram Coastal Fisheries Research and Development Center. After oyster collection, three oysters were initially used as negative controls. Remaining oysters were equally placed in 5 water tanks; 3 oysters each, all containing 10 L of sea water with salinity 30 ppt. One million *C. parvum* oocysts were seeded once to each tank at baseline. At specified time points: 24 h, 72 h, 7 days, 14 days and 30 days; 3 oysters from a tank were collected and processed for *C. parvum* detection and viability study with immunofluorescence (Sporo-Glo and Merifluor®) staining and confocal microscopy at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University.

2.3. Experiment 2: duration of viability and multiplication capacity

To determine the viability and multiplication capacity over 3 months, a further 52 similar commercial-size oysters were selected for experiment 2. In this experiment, four oysters were used as negative controls. Remaining oysters were hanged with a rope and equally placed in 4 water tanks (12 oysters each), each with 48 L of sea water with salinity 30 ppt. A total of 480000 oocysts were directly fed to the oysters and seeded to each tanks, every week for 12 weeks. After 7 days of each inoculations, 4 oysters were similarly sampled and processed for *C. parvum* viable oocysts and other stages detections, also by immunofluorescence (Sporo-Glo and Merifluor®) staining and confocal microscopy.

2.4. Sample processing

Samples were processed with procedures as previously described [10,11]. Initially, individual oysters were dissected; gills, digestive organs, and water inside the oysters were removed separately before being minced with 5 mL of PBS. Each part was placed in 50 mL tube and an additional PBS was added up to 20 mL. The suspension was filtered through 2 layers of wet gauze into a new 50 mL tube. Following an addition of 10 mL diethyl ether for sedimentation, the solution was shaken vigorously for 30 s and was centrifuged at 3500 rpm for 10 min. All supernatant was removed and 10 mL PBS was again added and centrifuged at the same speed prior to removing of the supernatant. The residual pellet was transferred to a 1.5 mL tube and was further centrifuged at 12000 rpm for 10 min. Finally, all supernatant was then removed and 200 μL PBS was added before vortex.

2.5. Immunofluorescence staining

A 50 μL of suspension were stained with IFA following both Koh *et al* procedures [6] and the manufacturer's protocols (Merifluor®, Meridian Bioscience Inc., OH, USA). First, samples were placed on a multi well slide that was partially air dried and fixed with 50 μL of ethanol for 8 min. Two hundred and fifty micro liters of 3% of BSA blocking buffer was added and allowed to stand for 30 min. The blocking was removed and completely replaced with Sporo-Glo antibody (1:100 in blocking buffers, Waterborne Inc., LA, USA) for staining viable oocysts and other stages of *Cryptosporidium*. The slides were placed in a humid chamber and incubated at 37 °C for 2 h. The humid chamber was removed from the incubator, and 10 μL of Merifluor® *Cryptosporidium/Giardia* was then added to the slides, for oocysts wall staining, and then kept in the chamber, which was left at room temperature for 1 h. Afterwards, the slides were rinsed with PBS and allowed to sit for 3 min. A 45 μL ProLong®-Gold Antifade Moutant with DAPI (Thermo Fisher Scientific, CA, USA) was then added to the slides for confocal microscopic examination.

2.6. Confocal microscope examination

All slides were thoroughly examined under confocal microscopy at 100× magnification (LSM 700, Zeiss, Germany) using the excitation and emission wavelengths of 495/517 for FITC, and 358/463 for DAPI nuclear staining. Samples were visualized and presented with Zen software (2009 edition, Zeiss, Germany), the number of oocysts, both viable and non-viable, ghost cells (empty oocyst), and other multiplication stages (if any) observed following the morphological references [6,12] were recorded and reported. Viability of the oocysts and other stages was assessed with the presence of complete oocysts wall filled with sporozoites or signs of excystation, and the viability rate of inoculated *Cryptosporidium* was calculated from the number of viable oocysts of all observed oocysts.

3. Results

3.1. Concentration and viability of Cryptosporidium oocysts in oysters

Following 24 h to 7 days after inoculation in experiment 1, *Cryptosporidium* oocysts were detected from digestive organs, gill and from water inside the experimented oysters, with the highest number observed at 24 h compared to other time points (Figure 1). Concentration and viability of oocysts in all parts of oysters decreased over time. At 24 h after seeding, we observed the highest number of oocysts in the digestive organs (Figure 1A) and were more viable than those observed in gill (Figure 1B) and water of inoculated oysters (Figure 1C). Viable oocysts were also seen in the digestive organs at 3 and 7 days after seeding (Figure 1A 3d, 7d), but less so in the gills and the water inside the oysters. Most oocysts in gill (Figure 1B 3d, 7d) and water of the studied oysters (Figure 1C 3d, 7d) were mainly non-viable. Due to the higher definition of staining with the Sporo-Glo antibody, the numbers of viable and non-viable of oocysts can be clearly identified to calculate the viability rate, which are approximately 70%, 60% and 30% at 24 h, 72 h and 7 days after exposure, respectively. No oocyst was detected in all parts after 14 days (Figure 2). Figure 2 also shows average number of *Cryptosporidium* oocysts in oysters at different time points, ranging from 24 h to 3 months after seeding. Similarly in experiment 2, the number of oocysts was lower with increasing time. There was an average of 857.33 oocysts found in oysters at 24 h, which then abruptly reduced to 243 and 126.67 oocysts at

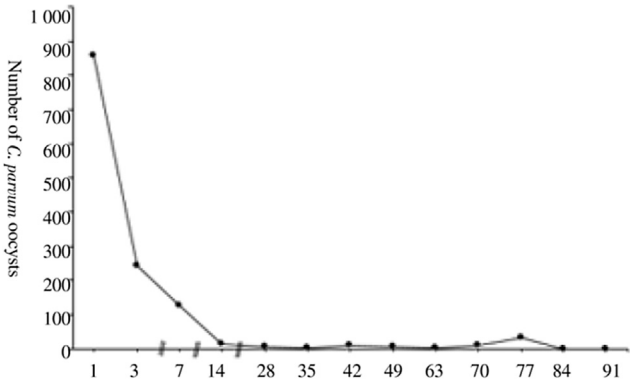

Figure 2. The average number of *Cryptosporidium* oocysts found in oysters at various times after inoculation from day 1 to day 91.
There were 857.33 oocysts in oysters after exposure of 24 h, then 243.00 and 126.67 oocysts were found at 3 and 7 day, respectively. Few number of oocysts ranged from 12.50 to 33.75 oocysts per oyster were detected after day14 to day77. No oocyst was found at day 84 and day 91.

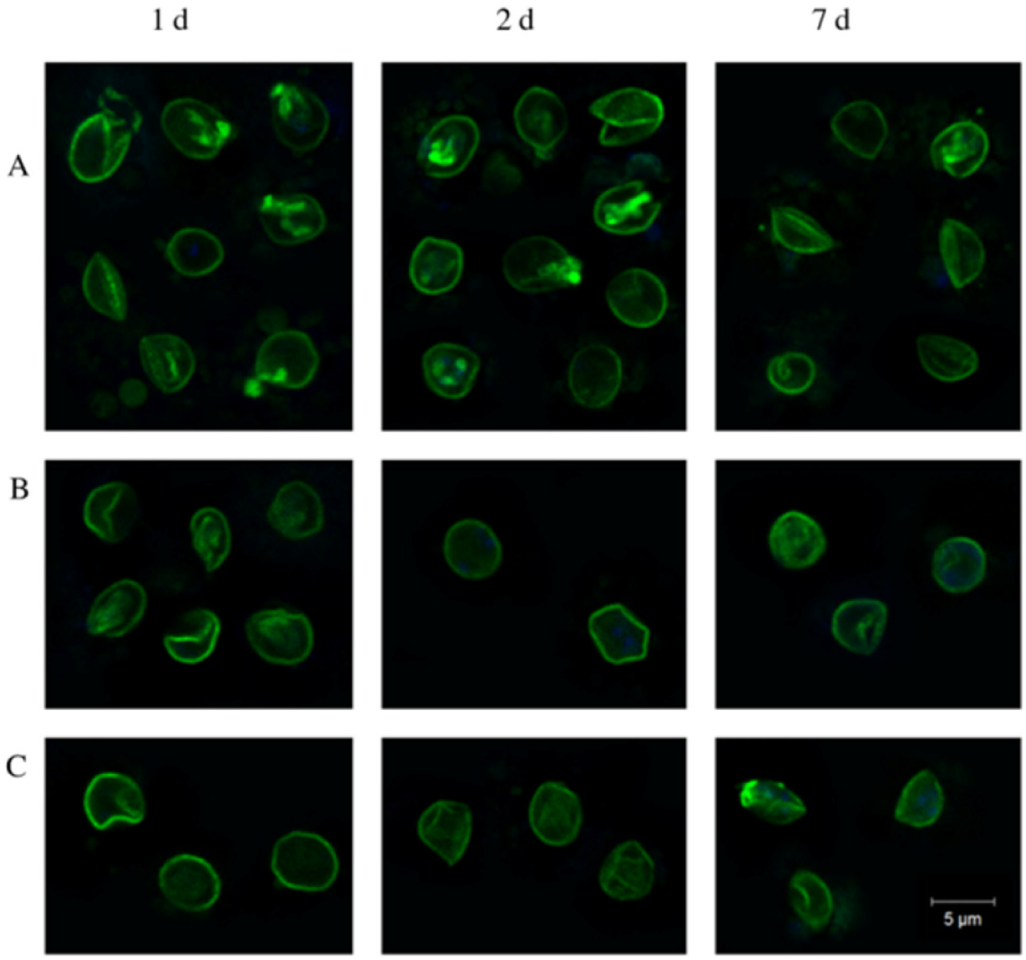

Figure 1. *Cryptosporidium* oocysts from digestive organ (A), gill (B) and water inside inoculated oysters (C) under confocal microscopic examination with immunofluorescence (Sporo-Glo and Merifluor®) staining.
Many viable oocysts with well-formed wall and containing sporozoites are present more in digestive organ at 24 h (A 1d) than 72 h (A 3d) and 7 days (A 7d), as well as more than other parts of oysters (B 1d, B 3d, B 7d and C 1d, C 3d, C 7d), which mostly contain ruptured oocysts wall with released sporozoites, and non-viable oocysts without sporozoites (ghosts cell); 1d = 24 h, 3d = 72 h, 7d = 7 days.

72 h and 7 days, respectively. Relatively few oocysts were detected after 14 days, and was no longer detected at 84 days. Despite the observed forms of *Cryptosporidium* oocysts and sporozoites found in the oysters, we did not find any other stages of *Cryptosporidium* multiplication throughout the study.

4. Discussion

In this study, *Cryptosporidium* oocysts can be taken up by oysters, mostly concentrated in digestive organ at all time points. This is perhaps due to the digestive organ being a cell morphologically comparable to the human intestine, and a nutrient reservoir suitable for *Cryptosporidium* survival than gill or water. The oocysts are 70% viable at 24 h after exposure, which dropped to 60% at 72 h, 30% at 7 days, and became completely absent after 14 days. The abrupt reduction in both oocysts number and viability rate seen after 24 h of inoculation and persisted for more than 3 days may be attributed to the depuration process in oysters, salinity and temperature of sea water [13].

Using Sporo-Glo antibody in combination with Merifluor® *Cryptosporidium/Giardia* oocysts wall staining clearly allows the detection of all stages of *Cryptosporidium* in oysters. Sporo-Glo antibody is intended for evaluating the viability of *C. parvum* oocysts, it is also typically used to detect sporozoites, merozoites, and other reproductive stages of *Cryptosporidium* [6], but has never been used in oysters. IFA remains the preferred method for routine analysis of *Cryptosporidium* oocysts in shellfish species [14]. It is reported that fluorescence diminishes overtime due to the action of hemocyst in oysters [15], we found that the combined technique illustrates more definite internal structure of oocysts than Merifluor® alone. The confocal microscopy further amplifies the resolution of the images and allows the digestive organ to be distinguished as the better transmission vehicle than gills and water in oysters.

A previous study by Freire-Santose *et al* [16] reported that oocysts in oysters (*Ostre edulis*) can remain infectious for 30 days. This contradicts with our results, which may be due different rate of depuration and hemocyst activity of different species of oyster, and higher water temperature in our study. Natural sea water was used in this study, which may contain some rotifers, ciliates and amoeba. Rotifer feeds on *Cryptosporidium* oocysts at a maximum of 25 oocysts/rotifer [17], while ciliates, such as paramecium, demonstrated the highest mean ingestion rates of up to 170 oocysts/h [18] and amoeba can feed on 3 *Cryptosporidium* oocysts per cell [19]. Therefore, it is possible that the oysters may be outcompeted by other microorganisms present in natural sea water that also feed on the oocysts. Salinity and temperature have been described as important factors affecting oocysts viability [20–23]. Fayer and colleagues showed the strong synergistic interaction between salinity and temperature where the oocysts remain infectious at 20 °C for 12, 4 and 2 weeks at salinity 0 and 10 ppt, 20 ppt, and 30 ppt., respectively [24]. Temperature was reported the most lethal factor affecting oocysts in the environment [25]. Since we aim to replicate the natural environment where oysters naturally grow, natural sea water with salinity at 30 ppt and temperature between 28 and 31 °C were used, thus may shorten the viability of oocysts.

This study is the first to confirm that *C. parvum* does not multiply in oysters, and is therefore not a biological host. Nevertheless, the result suggests that oyster can be an effective transmission vehicle for *Cryptosporidium* oocysts, especially within 24–72 h of contamination, with viable oocysts present at up to 7 days post infection.

Considering the high number of viable oocysts found at 24 h and 72 h which is sufficient to cause the infection, and raw oyster consumption still being more socially preferred than cooked oysters, cryptosporidiosis remains a public concern. For the safety of consuming oysters, it is necessary for commercial oysters to undergo depuration in clean sea water for 7 days or at least 1–3 days before consumption to remove viable *C. parvum* oocysts, and cooked oysters should be promoted as a safer alternative.

Declare of interest statement

We declare that we have no conflict of interest.

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