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USE OF *ARTEMIA FRANCISCANA* AS A BIOFILTER FOR CATCHING *CRYPTOSPORIDIUM PARVUM* OOCYSTS

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Summary

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Brine shrimp *Artemia franciscana* is capable to filter-feed on oocysts of *Cryptosporidium parvum*. In this study we have caused intake of oocysts by metanauplii of *Artemia* in concentration 4×10^3/mL. Our experiments also proved the possibility of intake of oocysts in the Sheather and Fullerborn solutions. We also caused intake of oocysts by metanauplii located in larger volume of water by this concentration of oocysts which will allow us to use metanauplii as oocysts detectors in further studies.

Key words: *Artemia franciscana*, *Cryptosporidium parvum*, oocysts, water

INTRODUCTION

Cryptosporidium parvum is an important protozoan parasite that is capable of causing gastrointestinal illness cryptosporidiosis in humans and animals (O'Donoghue, 1995). It can cause an acute but self-limiting gastroenteritis in immunocompetent people, while in immunocompromised patients the symptoms are particularly severe and cryptosporidiosis can be fatal (Striepen, 2013). *Cryptosporidium parvum* is spread by the faecal-oral route, either by ingestion of contaminated water or food or by zoonotic or human to human transmission (Xiao, 2010). Waterborne transmission is one of most important routes as it is responsible for numerous outbreaks worldwide (Baldursson & Karanis, 2011). *Cryptosporidium parvum* infectious stages – oocysts – are easily spread through water and are extremely hardy and resistant to various form of water disinfection which makes them difficult to remove from water (Fayer *et al.*, 2004).

Brine shrimp *Artemia franciscana* is a micro-crustacean, commonly used as live food in aquaculture. *Artemia* naturally occurs in hypersaline habitats and is a non-selective particle filter feeder. It develops in a form of larva, where metanauplius larva is a first stage in its life cycle when it starts filter-feeding (Lavens & Sogeloos, 1996). Méndez-Hermida *et al.* (2006) proved that *Artemia* metanauplii are capable of ingestion and excretion of *Cryptosporidium* oocysts. In another

study, Méndez-Hermida *et al.* (2007) established that oocysts remain viable even after intake by metanauplii, therefore they can serve as a potential cause of infection for fish or other susceptible host.

In this study we tried to ascertain what time is necessary for the intake of oocysts by metanauplii as well as selection of optimal solution for the intake of *Artemia*. In the second experiment we tried cause intake of oocysts by *Artemia* in a larger volume of water to ascertain if metanauplii are capable to filter-feed oocysts even in those conditions.

MATERIAL AND METHODS

Oocysts were obtained from faeces of naturally infected calf with the use of Sheather flotation according to Kuczynska & Shelton (1999) and Fullerborn salty water technique according to Göz *et al*. (2006).

This faecal sample was acquired from the calf with profuse diarrhoea after rectal sampling, where it was diagnosed with *Cryptosporidium parvum* genotype IIaA17G1R1 infection in different study (Hatalová *et al.,* 2017). One-hundred µL of feces were mixed with small amount of water, homogenised and filtrated through gauze. Sample was centrifuged at 500×g for 5 minutes and supernatant was poured out. Sediment was mixed with small amount of Sheather solution in one tube and Fullerborn salty water solution in the other. Subsequently, both tubes were filled with Sheather solution and Fullerborn solution accordingly. Tubes were centrifuged at 500 g for 5 min. Oocysts located at the top of the solution were extracted and enumerated yielding approximately 4×10^3 oocysts in 1 mL in both cases.

The experiment was carried out on *Artemia franciscana* hatched in sea-water of the following composition: NaCl 23.900 $g\cdot L^{-1}$, $MgCl_2\cdot 6H_2O$ 10.830 $g\cdot L^{-1}$, $CaCl_2\cdot 6H_2O$ 2.250 $g\cdot L^{-1}$, KCl 0.680 $g\cdot L^{-1}$, $Na_2SO_4\cdot 10H_2O$ 9.060 $g\cdot L^{-1}$, $NaHCO_3$ 0.200 $g\cdot L^{-1}$, $SrCl_2\cdot 6H_2O$ 0.040 $g\cdot L^{-1}$, KBr 0.099 $g\cdot L^{-1}$, H_3BO_3 0.027 $g\cdot L^{-1}$; pH 8.31 (Dvořák *et al.*, 2005). The *Artemia* larvae were raised until metanauplii (Instar V), which were used in two sets of experiments.

In the first set of experiments we decided to determine the time that is necessary for the intake of *Cryptosporidium oocysts* by *Artemia* metanauplii. For this experiment we used two sets of Petri dishes with 20 ml of 4% salty water with 20 *Artemia* metanauplii in which we added oocysts in 100 µl of Sheather solution in one set of Petri dishes and 100 µl of Fullerborn salty water solution in the other and stored in thermostat at constant temperature 24°C. We also used metanauplii without addition of oocysts as negative control for this experiment. *Artemia* were collected after 6, 12 and 24 hours after addition of oocysts, during which time we also determined viability of *Artemia* metanauplii.

After the collection of *Artemia* metanauplii, these were repeatedly washed in the sterile water to assure that oocysts are inside the metanauplii. Subsequently, the genomic DNA was extracted from the metanauplii using the DNA-Sorb-AM Nucleic acid Extraction kit (AmpliSence) according to the instructions given by producer. Before the extraction, the samples were homogenized and oocysts disrupted at 6500 rpm for 90 seconds with addition of 1.0-mm zircon beads, 0.5-mm glass beads and lysis solution in homogenizer Precellys 24 (Bertin technologies). After isolation the DNA was used in the Nested PCR reactions with primers GP60 F1 (5´- ATGAGATTGTCGCTCAT

TATC) /R1 (5´- TTACAACACGAATA AGGCTGC) in the first reaction and with primers GP60 F2 (5´- GCCGTTCCACT CAGAGGAAC) /R2 (5´- CCACATTAC AAATGAAGTGCCGC) in the second reaction. PCR products obtained after second reaction were analysed by electrophoresis in 1.5 % agarose gel and visualised by UV light with wavelength 312 nm. Samples that were positive were send for DNA sequencing.

In the second set of experiments we decided to determine the possibility of causing intake of oocysts by *Artemia franciscana* metanauplii that are located in the larger volume of water with the same dose of oocysts as that used in the first experiment. In this experiment we used oocysts prepared with Fullerborn salty water flotation technique. We used 1 L and 5 L of 4% salty water, where we put 500 metanauplii (±20) and 2,500 metanauplii (±200) respectively. After that we added the same dose and concentration of oocysts as mentioned above. After 24 hours we extracted the DNA, ran PCR reactions and visualised samples with electrophoresis as described above. Positive samples were sent for sequencing.

RESULTS

In the first round of experiments all samples collected after 6 hours and all samples from metanauplii without added oocysts were negative, while those tested after 12 and 24 hours were positive. This applies for both sets, the set with Sheather solution as well as the one with Fullerborn salty solution. Viability rate was higher in the *Artemia* with the Fullerborn salty solution (85%) than in those with Sheather solution (60%). In the second set of experiments both samples were identified as positive. All positive samples were determined to be *Cryptosporidium parvum* genotype IIaA17G1R1 which is identical as the genotype detected from the original calf sample.

DISCUSSION

Only two experiments with intake of *Cryptosporidium oocysts* by the brine shrimp *Artemia* were done before. Both of these were conducted by Méndez-Hermida, Gómez-Couso and Ares-Mazás, one in 2006 and the other in 2007. These authors used a large number of oocysts that were added to *Artemia* (1×10^4/mL and 1×10^6/mL), but we proved that intake of oocysts by *Artemia* is possible with even 4×10^3/mL. They also used various time intervals for the intake of oocysts by of *Artemia*, but haven´t specified which one of them is optimal, where we found out that with concentration 4×10^3 oocysts per 1 mL we are capable to cause intake of oocysts by *Artemia* in 12 hours. While Méndez-Hermida *et al.* used either formalin-fixed oocysts or oocysts prepared with Percoll gradient and repeated washes with PBS, we discovered that even such simple oocysts preparation methods like Sheather flotation and Fullerborn salty water technique can be used to cause intake of oocysts by metanauplii. We were also capable to cause intake of oocysts by *Artemia* metanauplii in the larger volume of water with as much as 100 µL of concentrated oocysts. This can be very useful in the future as it will allow us to use *Artemia* metanauplii for possible filtration of water collected from various sources such as lakes or rivers.

CONCLUSIONS

In this study we have confirmed that intake of *Cryptosporidium* oocysts by *Artemia franciscana* is indeed possible and

it can do so in the concentration of $4x10^3$/ml. The 100 µl of solutions with oocysts is even enough for the intake of oocysts by metanauplii in 5 liters of water. This experiment is important because metanauplii can be used as some sort of water filter that will allow us to check the presence of *Artemia* in various types of water.

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