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José Mijail Campos Compean
Maestría en Ecología Aplicada, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

Jaime Bustos-Martínez
Laboratorio de Microbiología y Biología Molecular Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

Ignacio Barbabosa Martínez
Laboratorio de Microbiología y Biología Molecular, Departamento de Atención a la Salud Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

María del Carmen Monroy Dosta
Laboratorio de Análisis Químico del Alimento Vivo para la Acuicultura. Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

Aida Hamdan-Partida
Laboratorio de Microbiología y Biología Molecular, Departamento de Atención a la Salud Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

Correspondence
José Mijail Campos Compean
Maestría en Ecología Aplicada, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso, Col. Villa Quietud. Coyoacán, Ciudad de México

Detection and typing of *Blastocystis* spp. in oysters (*Crassostrea virginica*) collected in Actopan River, Chachalacas, Veracruz

José Mijail Campos Compean, Jaime Bustos-Martínez, Ignacio Barbabosa Martínez, María del Carmen Monroy Dosta and Aida Hamdan-Partida

Abstract
The main goal of this study was to determine the presence and *Blastocystis* spp subtype in oysters (*Crassostrea virginica*) collected at Actopan River, Veracruz. Three samplings were made during one year at the principal oyster collection areas. The oysters were dissected to obtain their gut, which was inoculated in *Blastocystis* spp. culture medium and incubated for seven days to analyze them by microscopy technique. Samples that were positive to *Blastocystis* spp. their DNA was extracted by PCR technique and amplified ITS1–5.8S–ITS2 region of 18S rDNA gen. The amplicons were sequenced and subsequently were typing using bioinformatic program. It was found that *C. virginica* show a high prevalence of *Blastocystis* spp., 67% were positive and it was determined that *Blastocystis* spp. subtype isolated from *C. virginica* was subtype 1 (ST1).

Keywords: *Blastocystis* spp, *Crassostrea virginica*, parasite, typing

1. Introduction
In México, oyster activity has significant role in country aquaculture production [8], because it is a food source of high nutritional value, employment generating industry, and economic and foreign exchange income [3]. However, mollusks collection is affected by anthropogenic disturbance that pollute natural environment with chemicals and fecal matter [10]. The oysters are filtered organisms that ingest into their guts organic matter what can be polluted with infection agents like: virus, bacteria, and parasites, so they can be potential pathogen reservoirs to human being, especially because oysters tend to be raw consumed, implying a risk to consumers [2]. Within protozoa parasites reported in *C. virginica* can be mentioned: *Nematopsis prytherchi* and *Perkinsus marinus* [1, 9]. However, *C. virginica* is candidate to be reservoir of *Blastocystis* spp. because is a worldwide parasite whose usual location is the human's digestive system, and of other animals, it is transmitted by water or polluted foods with fecal matter [4]. It is a facultative anaerobic unicellular protozoa, with retractile body and diameter between 4 a 15 μm, and possess four vegetative forms: central body, granular, ameboid, and cyst [19]. It is known 17 subtypes of *Blastocystis* spp. depending on animal species that parasite, subtypes 1 to 9 are found in humans and some are share with other animals, meanwhile, subtypes 10 to 17, are reported in primates, rodents, birds, reptiles, and snakes [16]. Due to above, the main goal of this study is to determine the presence and subtype of *Blastocystis* spp. isolated in specimens of *C. virginica* collected in Actopan River mouth, Chachalacas, Veracruz.

2. Materials and Methods
2.1 Samples collection
C. virginica samples were collected by autonomous diving at Actopan River mouth, Úrsulo Galván municipality, Chachalacas, Veracruz. The geographical area is in central zone of Veracruz State at coordinates 19° 24' LN and 96° 18' LW. Three samplings were made for one year; first one was in June 2016 obtaining 100 organisms with fisherman assistant; second sampling was made in February 2017 (100 organisms); and third sampling was made in October 2017 and were obtained 50 organisms. The oysters were processed and analyzed in the Microbiology and Molecular Biology Laboratory of Atención a la Salud Department from

UAM-Xochimilco.

2.2 Vegetative forms cultures to *Blastocystis* spp. identification

Oysters were dissected to obtain the gut from each specimen and introduced in test tubes with *Blastocystis* spp. culture medium, with NaCl solution at 0.85%, fetal bovine serum at 10%, and covered with one drop of mineral oil. Test tubes were incubated at 37°C for seven days.

2.3 Microscopical analysis

Each gut samples were examined by microscopy (20X and 40X), searching for distinct stages of *Blastocystis* spp. determining their abundance by direct counting with Neubauer chamber, and comparing with different reference images [11, 18, 19] to identified *Blastocystis* spp. correctly.

2.4 Microscopy data processing

With generated data was made a database in Excel 2017, and processed in Prism 6 program to obtain prevalence and abundance graphs.

2.5 *Blastocytis* spp. identification by PCR technique

To make parasite identification, was use method described by Villalobos [16], which consisting to amplified the ITSI-5.8S.ITS2 region of 18S rDNA gen using PCR with following primers: ITS_Blas_F (5′-GGA AGG TGA AGT CGT AAC AAG-3′) and ITS_Blas_R (5′-CAG CAG GTC TTC TTR CTT GA-3′), which were amplified a fragment of ~550 to subtype 1, ~530 to subtype 2, ~620 to subtype 3, and ~590 pb to subtype 7. PCR mixing volume was of 20 µL and contain: dNTPs 1 mM, magnesium chloride 2 mM, 5X colorless GoTaq flexi buffer, bovine serum albumin $1.5X10^{-4}$ mM, ITS_Blas_F primer 0.5 mM, ITS_Blas_R primer 0.5 mM, 1 U of GoTag DNA polymerase and 50 µg of DNA sample. With BioRad thermocycler Myclycler, was made amplification with following conditions: initial denaturalization at 94º C for 30 seconds, later 35 cycles of denaturalization at 94º C for 30 seconds, annealing at 60ºC for 45 seconds, extension at 72º C for 30 seconds and final extension at 72° C for 10 minutes. The amplicons were submitted to electrophoresis agarose gels at 1.2%, and after were purified with ExoSAP (New England de UK) kit and send to sequencing to Macrogen Korea.

2.6 *Blastocystis* spp. typing isolated of *C. virginica*

Obtained sequences were submitted to Blast search in database of National Center of Biotechnology Information). Subsequently, published sequences were downloaded in the Genbank of ITS1–5.8S–ITS2region from 18S rDNA gen to *Blastocystis* spp. subtypes 1, 2, 3, and 7, founded in humans [16], and with *Blastocystis* spp. sequences founded in *C. virginica*, were made multiple alignments using ClustalX program. Then, was used Seaview program to cut leftover sequences and with MEGA7 program was determined identity percentage, and *Blastocystis* spp. was typing whit rounding shape typing tree construction with maximum likelihood tree method.

The obtained sequences of *Blastocystis* spp. in *C. virginica* were entered to Genbank with MG921687 and MG921688 numbers.

3. Results

From microscopic analysis in *C. virginica* it was determined the prevalence for each sampling period. There was not observed important variations between periods, because it was obtained a prevalence of 69% for first period, 60% for second and 74% for third (Fig. 1).

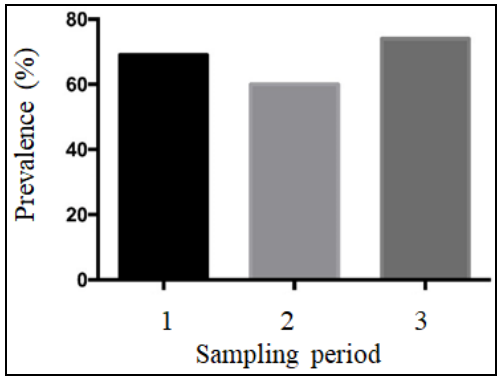

Fig 1: Prevalence of *Blastocystis* spp in *C.virginica* in Chachalacas Ursulo Galvan municipality, State of Veracruz.

Regarding to different stages abundance of *Blastocystis* spp in analyzed samples, only can observed the central body or vacuolar (Fig. 2).

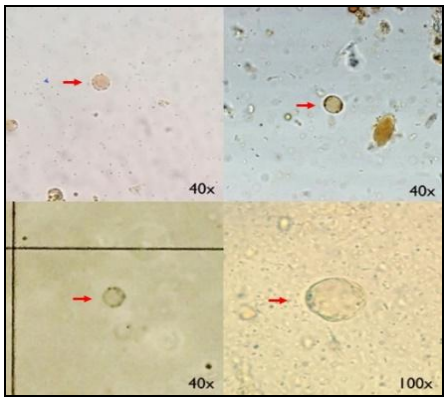

Fig 2: Central body or vacuolar of *Blastocystis* spp isolated from *C. virginica*

In first sampling it was observed a total of 342 central bodies, with a mean of 5 ± 1.7 central bodies per analyzed sample. For second sampling, it was counted 281 central bodies with a mean value per sample of 4.7 ± 2.2 and for third sampling only 50 samples were obtained, in which 147 central bodies were counted with a mean value of 4.1 ± 1.5 per sample (Fig.3)

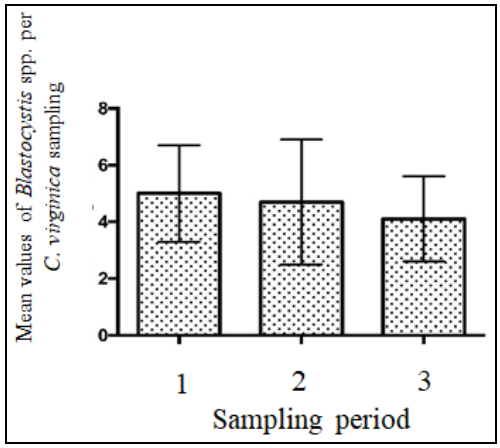

Fig 3: Mean values of *Blastocystis* spp abundance per sample of *C. virginica*

Amplification of ITS1–5.8S–ITS2 region of 18S rDNA gene with primers: ITS_Blas_F e ITS_Blas_R, allowed to obtain PCR products of ~550 bp, which correspond to expected size of amplicon for *Blastocystis* spp (Fig. 4).

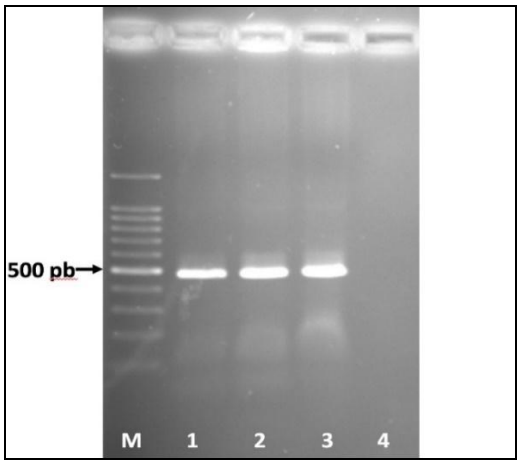

Fig 4: Amplification of ITS1–5.8S–ITS2 region of 18S rDNA gene. Agarose gel at 1%. M) Molecular marker of 100-1000 bp 1) and 2) *C. virginica* digestive tube samples 3) Positive control 4) Reaction control

From phylogenetic analysis it was obtained a typing tree, which shown that *Blastocystis* spp. subtype isolated form *C. virginica* belong to 1A, as observed in red boxes (Fig. 5).

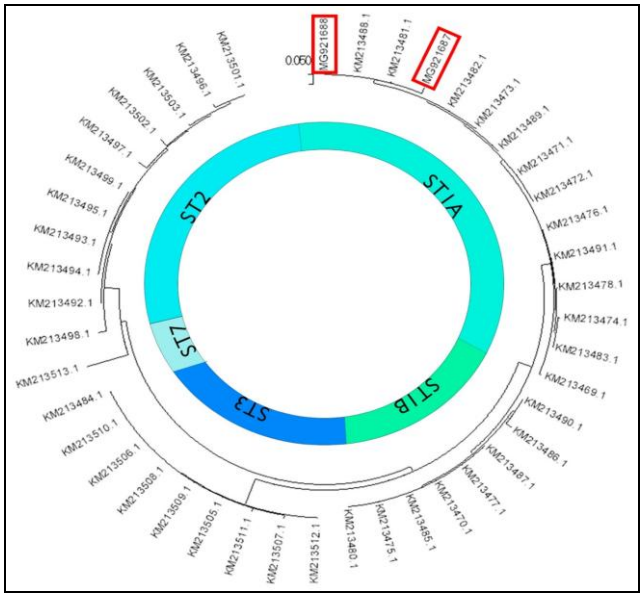

Fig. 5: *Blastocystis* spp. typing isolated from *C. virginica* from ITS1–5.8S–ITS2 region of 18S rDNA gene and archived in Genbank [16]

4. Discussion

In River Actopan, located in Chachalacas, Veracruz there is a people community which are working on *C. virginica* collection. It is known that these areas are being affected by chemical and fecal matter contamination [12], moreover, Chachalacas beach is an area with a high tourism presence, where is common the sale and consumption of raw oyster, implicating a risk of *Blastocystis* spp. infection for their consumption.

In this work it was detected *Blastocystis* spp. parasite presence with a prevalence of 67%, which is similar to those value reported in previous study [11] with a 77% prevalence. *Blastocystis* spp. prevalence can vary between seasons [13], but in *C. virginica* it was identified in more than 50% of collected organisms in all samples. This shows parasite presence in oysters between seasons and its prevalence is independently to environmental factors, so the principal variable that determines *Blastocystis* spp. presence in *C. virginica* is polluted water medium where oysters were collected. Several studies mention that in Veracruz coastline intertidal zones are crowded systems, because they provide environmental services of recreation and maintenance [6], but show polluted problems because water discharges produced by high touristic activity and coast line urban spot [10, 14].

In previous studies, it has been reported that *Blastocystis* spp. subtype that is most often founded in human being was ST1, followed by ST3 [14]. The obtained results in this study by typing technique determined that identified *Blastocystis* spp. bodies correspond ST1 subtype, so it can be deduced that its presence in oyster is due to continuous discharge of feces in River Actopan mouth [15]. Nevertheless, it has also been reported subtype 1 in cattle [7] and in Úrsulo Galvan municipality which have 15,941 hectares dedicated to cattle raising with 6,178 cattle heads [5], therefore generalized use of cattle raising and agriculture in this region associated to river margins, increase protozoa presence [17], so it can be considered, in general way, there is polluting waste poor handling that provides fecal matter from various sources [18].

All the above, it is required immediately an improvement in water discharge handling in River Actopan mouth in order to avoid a public health problem associated to consumption of mollusk bivalve.

5. Conclusion

The recollected oysters (*C. virginica*) in Chachalacas, Veracruz zone are colonized with *Blastocystis* spp. subtype 1, which probably is due to animal and human fecal contamination that River Actopan shows but, it is important to generate actions against River Actopan pollution to eliminate *Blastocystis* spp. presence in *C. virginica*.

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