ARTÍCULO DE INVESTIGACIÓN

Identification of *Blastocystis* ST1 in oysters (*Crassostrea virginica*) collected in self-service markets in Mexico City

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Abstract. *Blastocystis sp. is an intestinal host of the digestive tract of a great variety of vertebrate and invertebrate organisms. The objective of this study was to determine the presence of Blastocystis sp. in American oysters () and to identify its subtype. Stools of 550 oysters obtained from self-services markets in Mexico City were analyzed with photon microscopy by means of direct examination in fresh feces colored with Lugol solution. DNA from the intestinal content of a group of oysters was isolated to detect the presence of Blastocystis sp. and to determine its subtype, by means of PCR amplification of the ITS1-5.8S-ITS2 region. The amplicons were sequenced and later typed by bioinformatic analysis. Cysts or vacuolar forms of Blastocystis sp. in 71.3% of the oysters were observed by light microscopy. The Blastocystis subtype (ST) identified by PCR in C. virginica corresponds to the ST1 subgroup A. We conclude that consumption of these raw oysters is a possible source of infection of Blastocystis sp. for humans.*

Keywords. *Blastocystis, ST1, C. virginica, Oysters, Self-service markets.*

Resumen. *Blastocystis sp. es un huésped intestinal del tracto digestivo de una gran variedad de organismos vertebrados e invertebrados. El objetivo de este estudio fue determinar la presencia de Blastocystis sp. en ostras americanas (Crassostrea virginica) e identificar su subtipo. Se analizaron las heces de 550 ostras obtenidas de tiendas de autoservicio en la Ciudad de México, con microscopía de fotones mediante un examen directo de heces frescas coloreadas con solución de*

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Lugol. Se aisló el DNA del contenido intestinal de un grupo de ostras para detectar la presencia de Blastocystis sp. y determinar el subtipo al que pertenece, mediante la amplificación por PCR de la región ITS1-5.8S-ITS2. Los amplicones se secuenciaron y luego se tipificaron mediante análisis bioinformático. Se observaron quistes o formas vacuolares de Blastocystis sp. en el 71.3% de las ostras por microscopía óptica. El subtipo (ST) de Blastocystis identificado por PCR en C. virginica corresponde al ST1 subgrupo A. Concluimos que comer ostras crudas es una posible fuente de infección de Blastocystis sp. para los humanos.

Palabras clave. *Blastocystis, ST1, C. virginica, Ostras, Mercados de autoservicio.*

INTRODUCTION

Blastocystis sp. is an intestinal protist that belongs to the Chromista kingdom, located in the Stramenopiles sub-kingdom. Besides being a common organism of the human intestinal tract, it is found colonizing the intestine of a wide variety of vertebrates (cats, pigs, dogs, poultry, rodents and even cockroaches), and is considered a zoonotic protozoan (Scanlan, 2012; Stensvold *et al*., 2007; Parija and Jeremiah, 2013; Zierdt *et al*., 1967).

Blastocystis sp. are facultative anaerobic organisms, it presents from one to four nuclei and typical organelles of a eukaryotic cell. They usually present four different forms: vacuolar, granular, ameboid and cyst. The vacuolar form also called central body is characterized by a large central vacuole that occupies most of the cell, surrounded by a peripheral band of the cytoplasm with one or more nuclei. Its size varies from 3 μm to 120 μm. It has a surface coating that is rarely observed to protect it from osmotic shock, and it is has been postulated that it participates in the capture of bacteria for its nutrition. The granular form is similar to the vacuolar form, except for the presence of metabolic, reproductive or lipid granules. The diameter of the granular forms ranges between 15 μm and 25 μm. The amoeboid form (trophozoite) presents pseudopodia and has a large phagocytic activity. It has an irregular shape and generally measures around 10 μm in diameter. The shape of the cyst is spherical and smaller than the vacuolar and granular form. Cysts isolated from humans usually have a diameter of 3-6 μm and do not exceed 10 μm, while larger cysts have been isolated from animal hosts. The cyst is the only transmissible form of *Blastocystis* sp., it confers protection during adverse conditions and it is considered to remain viable up to 1 month at 25 °C even to air exposure (del Coco *et al*. 2017; Martínez-Barbabosa *et al*., 2017; Stensvold *et al*., 2007; Zierdt, 1967).

The epidemiology of *Blastocystis* sp. is somewhat enigmatic and controversial: while the source of the infection and the mechanism of transmission are not yet known with precision, the most accepted explanation is fecal-oral as that of other intestinal protozoa (transmitted by fecalism). Humans can acquire zoonotic infections, and especially *Blastocystis* sp. due to the great variety of hosts that it has. However, the possibility of transmission of this chromist by the consumption of oysters infected by *Blastocystis* sp. has not yet been satisfactorily investigated, especially in oysters distributed in supermarkets. The present study was carried out as an attempt to fill this gap. The main objective of this work was to detect the presence of *Blastocystis* sp. among the bivalve mollusk *Crassostrea virginica* collected in self-service markets, as a possible source of infection for humans.

MATERIAL AND METHOD

Sampling

In a period of four months (June to September 2018), a cross-sectional exploratory descriptive study was carried out that consisted in the collection of 550 American oyster *C. virginica* for the search of *Blastocystis* sp. The oysters were obtained by simple random sampling in different self-service markets in Mexico City.

Microscopic analysis

Each mollusk was placed in a sterile petri dish to obtain the intestine by dissection. The intestinal content was analyzed by direct examination in fresh with 0.85% sterile NaCl solution and Lugol solution. The morphological identification of *Blastocystis* sp. was done with Carl Zeiss photonic microscopes at 100X and 40X magnifications. Fecal smears stained with Gomori were made for fine observation of the morphology of *Blastocystis* sp. (Martínez-Barbabosa *et al*., 2017).

Molecular identification of *Blastocystis* sp.

Total DNA was obtained from the intestinal content of a sample of 15 oysters, applying the Guanidine Thiocyanate technique. Briefly, a lysis solution containing 5M guanidine

ticianate, 10mM EDTA, 50mM Tris, 2% lauryl sarcosyl is added and subsequently the DNA is precipitated with 3M sodium acetate at pH 5.2, finally lipid and protein residues are eliminated with a chloroform-isoamyl alcohol solution (24: 1) (Sambrook and Russell, 2001). The detection and identification of the subtype (ST) of *Blastocystis* was carried out using the methodology described by Villalobos et al. (2014), which consists in amplifying the Internal transcribed spacer (1 and 2) plus the 5.8S region (ITS1+5.8S+ITS2) of rDNA by PCR. Using the following primers: ITS-Blas-F (5'-GGA AGG TGA AGT CGT AAC-3 'AAG), ITS-Blas-R (5'-CAG GTC TTC TTR CTT GA-3 '), which amplify a ~ 550 bp fragment to ST 1, ~ 530 bp to ST 2, ~ 620 bp to ST 3 and ~ 590 bp to ST 7. The mixed volume of the PCR containing: 1 mM dNTPs, 2 mM magnesium chloride, 5X colorless GoTaq flexi, $1.5X10^{-4}$ mM bovine serum albumin, 0.5 mM primers, 1 U of GoTaq DNA polymerase (Promega, USA) and 50 μg of DNA sample. A thermocycler Myclycler (BioRad, USA) was used, the amplification conditions were an initial denaturation at 94 °C for 30 s, 35 cycles after denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min. The amplicons were electrophoresed on 1.2% agarose gels. Positive samples were purified with the ExoSAP kit (New England, UK). The purified amplicons were sent to sequence to Macrogen, Korea.

To determine the subtype of *Blastocystis*, the sequences obtained from the oyster samples were analyzed using the BLAST database of the National Center of Biotechnology Information. The sequences were registered in GenBank obtaining the registration numbers MG921687 and MG921688. Subsequently, sequences from the ITS-5.8S-IT2 region of subtypes 1, 2, 3 and 7 of *Blastocystis* found in humans were downloaded from GenBank. These sequences were aligned with the sequences found in *C. virginica* using the Clustal X program. Finally, through the program MEGA 7 a phylogenetic tree was constructed with Maximum Likelihood method to determine the subtype to which it belongs.

RESULTS

The total microscopic analysis of the oysters showed the presence of cysts or vacuolar form of *Blastocystis* sp. in 71.3% of the samples. Figure 1 illustrates the cyst form of *Blastocystis* sp. dyed with Lugol, inside there are granules. The vacuolar form of *Blastocystis* sp. stained with Gomori was observed in Figure 2, it allows to appreciate a cell of around 10 μm in diameter, with a large central vacuole that occupies more than 70% of the cell surrounded by a peripheral band of the cytoplasm with several nuclei and numerous mitochondria.

Figure 1. *Blastocystis* sp. cyst in *C. virginica* feces stained with Lugol solution (40X magnification)

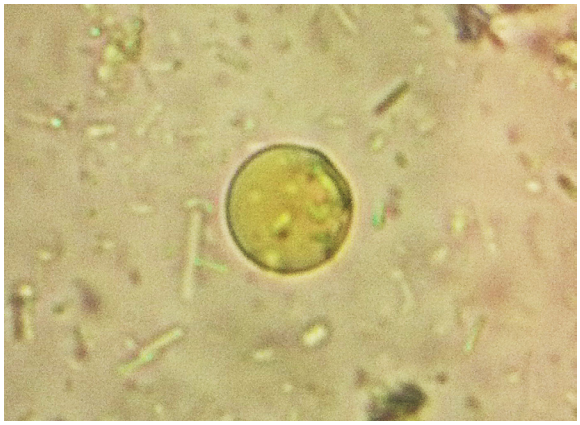
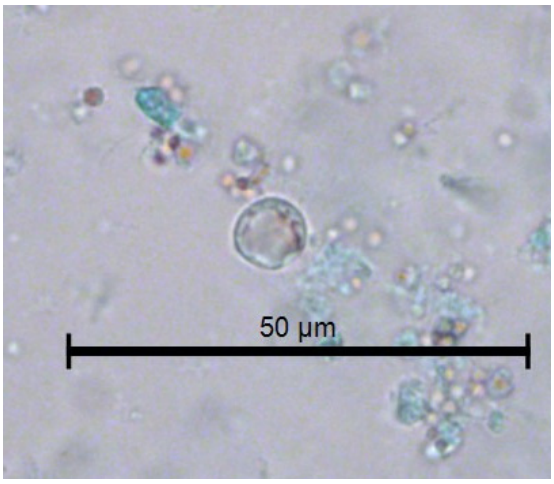


Figure 2. Image of the vacuolar form of *Blastocystis* sp. in stool of *C. virginica* stained with Gomori stain (100X magnification)



Molecular detection of *Blastocystis* by amplifying the ITS1-5.8S-ITS2 sequence in some of the oyster samples analyzed is observed in the Figure 3. It can be seen that samples C2 and C15 present an amplicon about 450 bp, like the control sample, which indicates the presence of *Blastocystis*, the other samples were negative. The amplicons were sequenced, presenting a size of 436 pb and when aligned in BLAST it presented a percentage of identity of 96% with *Blastocystis* sp. When making a phylogenetic tree using the ITS sequences of human *Blastocysitis* obtained from GenBank, we found that it belongs to the *Blastocystis* subtype ST1 subgroup A, Figure 4.

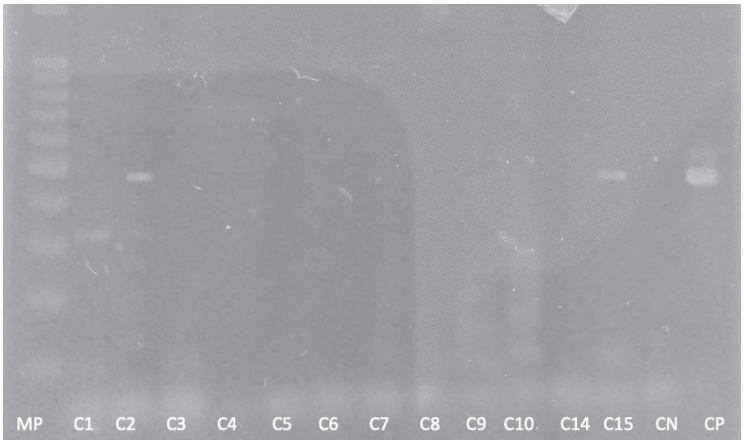
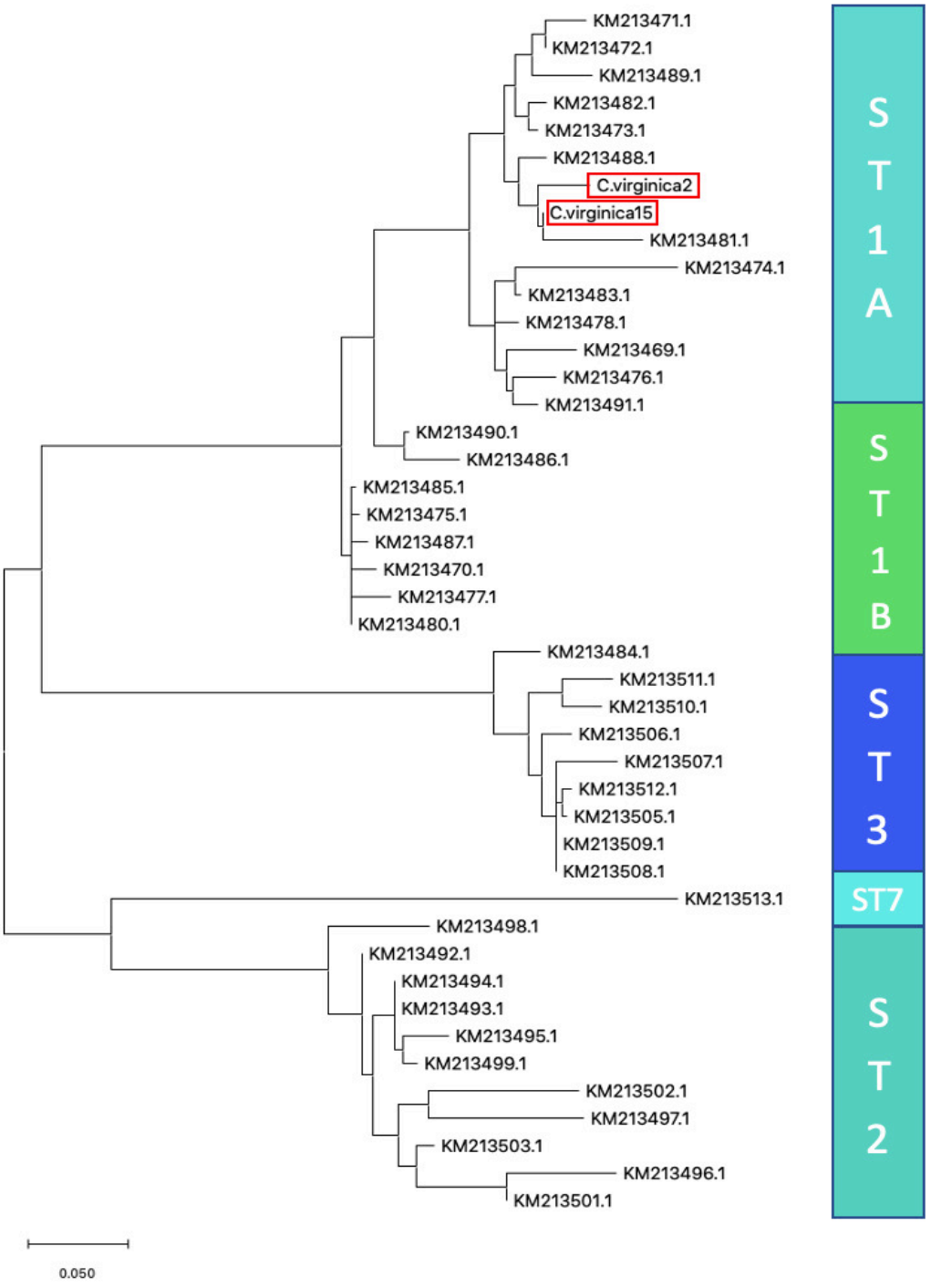


Figure 3. Amplification of the ITS1-5.8S-ITS2 region of rDNA by PCR. 1.5% agarose electrophoresis gel. C1 to C15: samples taken from oysters; MP: 100pb molecular weight marker; CN: Reaction control; CP: positive control sample of *Blastocystis* isolated from human.

Figure 4. Phylogenetic tree of *Blastocystis* STs using ITS1-5.8S-ITS2 region sequences. The phylogenetic tree was constructed with Maximum Likelihood method. The sequences of samples 2 and 5 isolated from *C. virginica* are included (MG921687 and MG921688), remaining in the clade of the subtype ST1 subgroup A.



DISCUSSION

In Mexico, the exploitation of oysters is one of the important fishing activities. The Gulf of Mexico generates 90% of the national oyster production. The American Oyster *C. virginica* is the most commercially important. It is distributed from the Gulf of San Lorenzo in Canada to the Laguna de Términos in the state of Campeche, Mexico. The largest production is carried out in the states of Tabasco and Veracruz. The annual oyster production is 400 thousand tons (Contreras and Castañeda, 2000), it is considered as an important oyster reserve in the American continent.

Oyster consumption is mainly due to its low price and high nutritional value, it provides 9.7% protein, 3.1% carbohydrates, and 1.7% fat plus vitamins A, B, C and D, same as phosphates, chlorides, and high zinc content. Basic nutrients in human nutrition.

The oysters are mollusks of the lamelibranch or bivalve group, to which numerous edible species belong. The oysters are fed by filtration, it is estimated that around 150 L of water per day are filtered, viruses, bacteria, protozoa, cysts, eggs and larvae of different organisms have been found so they can serve as potential reservoirs of pathogens for the human being, especially when consumed raw, which implies a risk for the consumer to develop important zoonoses such as blastocystosis (Aguirre-Macedo et al., 2007; Cabrera *et al*., 2010).

In recent years the prevalence of blastocystosis in the world population has increased significantly, to such a degree that it exceeds giardiasis and other protozoosis transmitted by fecalism (Boorom et al., 2008; Dudlová *et al*., 2018; Martínez-Barbabosa *et al*., 2010; Weerakoom *et al*., 2018).

From the epidemiological point of view, the consumption of raw oysters infected with *Blastocystis* sp. can be a risk to the consumer as has been already reported (Martínez-Barbabosa *et al*.,2017; Martínez-Barbabosa *et al*., 2018; Campos *et al*. 2018). Because this microorganism could adapt to the physiological conditions that gives the human intestine and thus get to reproduce and produce the infection. We found a high presence of carrier oysters of *Blastocystis* sp. by microscopic observation. Studies conducted in other species indicate that host specificity seems to have some relation to the *Blastocystis* subtype that colonizes it. It is not known if the oyster is colonized by *Blastocystis*, which should be investigated by determining the life cycle of this protozoan in oysters.

Studies of genetic diversity have led to the identification of numerous subtypes within the genus *Blastocystis* (Stensvold *et al*., 2007). So far, 26 subtypes have been identified (Stensvold and Clark, 2020), of which ST1 to ST8 colonize the intestine of humans and other hosts, ST9 only colonizes man, the other subtypes have not been identified

in humans. The ST3 subtype is the one most frequently detected in humans, but the ST1, ST2 and ST4 subtypes have also been regularly identified (Villalobos *et al*., in 2014, Parija and Jeremiah, 2013; Stensvold *et al*., 2012). In this work, we found the presence of *Blastocystis* of the subtype ST1, specifically from subgroup A, as proposed by Villalobos *et al*., in 2014, since after doing the bionformatic analysis the sequences found in the analyzed oyster samples are grouped into the clade of subgroup A of ST1 (Figure 4).

A possible cause of the presence of *Blastocystis* in oysters is human or animal fecal contamination in aquifers areas, but this has to be yet corroborated. If this is true is necessary to generate measures or actions that can mitigate the contamination of the aquifers and be able to eliminate the presence of *Blastocystis* sp. in *C. virginca*,

Due to these evidences, there is the possibility of using *C. virginica* as a surveillance model for *Blastocystis* infections in humans in coastal areas, that is, the presence of *Blastocystis* sp. in oysters it could indicate the possible presence of this protozoan in humans.

The presence of *Blastocystis* ST1 in oysters *C. virginica* implies that the corresponding sanitary authorities consider these oysters as a reservoir of *Blastocystis* sp. and to blastocistosis one of the emerging foodborne parasitic diseases (PTA) such as taeniasis, toxoplasmosis, trichinosis and hydatidosis (FAO / WHO, 2014). The pathogenicity of *Blastocystis* ST1 found in *C. virginica* will only be confirmed until the relevant studies are carried out. The association between the subtypes of *Blastocystis* and the clinical manifestations that can produce is still controversial, although there is some evidence to suggest that it behaves like a true pathogen (Dogruman-Al *et al*., 2009; Parija and Jeremiah, 2013; Stensvold *et al*., 2013;). Infection in immunocompromised persons can lead to severe diarrheal syndrome (Deepika *et al*., 2017; Paboriboune *et al*., 2014; Rasti *et al*., 2017).

Therefore, these results suggest that oysters are a possible source of infection for humans, hence it is advisable to cook the oysters before consumption.

CONCLUSIONS

Microscopic evidence indicates the presence of *Blastocystis* in *C. virginica* obtained from self-services markets. Likewise, the molecular analysis determined that *Blastocystis* ST1 subgroup A was found; this subtype can be found in the human population. These findings are important because the consumption of raw oysters with *Blastocystis* ST1 should be considered as a possible route of transmission for humans.

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DECLARATIONS OF INTEREST

None

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