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Presence of plasmid-R in isolated bacteria of oyster (*Crassostrea virginica*), during its process of collection, distribution, commercialization, and consumption

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Abstract
The aim of the present study is to determine presence of Plasmid-R in isolated bacteria of *C. virginica*, during its process of collection, distribution, commercialization, and consumption in Alvarado, Veracruz lagoon. After being identified, strains were subjected to processes of: alkaline extraction of Plasmid-R; electrophoresis in agarose gel; and to susceptibly to antibiotics determination, through plate diffusion. 34 different species of pathogenic bacteria for humans were identified, belonging to Enterobacteriaceae, Vibrionaceae, Aeromonadaceae and Pseudomonadaceae families, from which a total of 519 Plasmid-R were obtained, 37 in recollection, 87 in distribution, 203 in sale and 192 in the phase of oyster consumption. There was no significant difference in number of plasmids between each phase, nevertheless, the increase in number of Plasmids-R through the process of commercialization is significant. In each phase it was registered bacteria with resistance to antibiotics like ampicillin, carbenicillin, cephalothin and nitrofurantoin.

Keywords: *Crassostrea virginica*, enterobacteriaceae, plasmids-R, Veracruz

1. Introduction
Oyster farming activity in Mexico is considered one of the most important fisheries activity in the country [1], because it serves as a source of employment and income for population, supplies for food industry, and foreign exchange, also it promotes protection and conservation of endangered species [2]. Likewise, oysters are a highly nutritive food, its protein content represents the 50% of dry matter and it is totally digestible [3]. Nevertheless, oyster farming has several limitations, occasioned by overexploitation, contamination, siltation of coastal lagoons and sanitary problems [4]; alterations that sometimes have provoke exhaustion of oyster banks and also big problems in public health [5]. The most important oyster-farming centers of Mexico are located in coastal zone of Gulf of Mexico [6], which have in the surroundings industrial complexes and large population centers that pour into their waters many domestic, urban and rural wastes that affects oyster quality [7]. In aquaculture the accepted practice to control and prevent bacterial infections, is to supply antibiotics and other chemicals routinely, which enter to different aquatic systems through contaminated water of nosocomial, domestic, urban, suburban and rural origin [8]. Antibiotics make a "selective pressure" on bacteria and the strains that acquire resistance are the ones that survive and reproduce, so that the percentage of this microorganisms will increase, while sensitive strains disappear. Bacteria develops defense mechanisms as Plasmids-R as a response to antibiotics. Which have medical importance in bacterial resistance; pathogenicity or virulence; colonization of specific sites of host; and production of substances like toxins, enzymes and other molecules that make harm and are encoded by those plasmids [9]. So the aim of this study was determine the presence of Plasmid-R in isolated bacteria of oyster (*Crassostrea virginica*), during its process of collection, distribution, commercialization and consumption.

2. Materials and Methods
2.1 Microbiological analysis
In present study it was recollected 240 samples of oyster *C. virginica*, in three sampling sites **(**Manglar, Tío Luis and Arbolillo) in the lagoon system of Alvarado, Veracruz, (Lat N 18^0

44¨00´´ and 19^0 52¨15¨¨ and Long W 95º 44´00´´ and 95º 57´00´´), in distribution, sale and consumption zones of oyster in Mexico City, during the months of January-April, May-July, September-December (2007). Oysters were conserved in refrigeration at 4° C for its transfer to laboratory. Later on, oysters were shucked in sterile zone, and 10 g of sample were taking (per site and date) and were homogenized with 90 ml of sterile distilled water, during 3 min. Dilutions 1:10 until 10^{-7} were made. From each dilution with an automatic pipette, 1000 µl were extracted and inoculated by duplicate in agar plates with specific media of: *Salmonella- Shigella* (SS), Eosine-Methylene Blue (EMB), Thiosulfate Citrate Bile Salts (TCBS) and Brain Hearth Infusion Agar (BHI). Plates were incubated during 24 h at 36 °C to make the count of colony forming units per milliliter (cfu/ml) for each dilution. From the tube with the original homogenate was transferred 1000 µl of sample to tubes with lactose broth and tetrathionate broth, to which it was added 1000 µl of iodine iodide and transferred to a tube with peptone water and were incubated during 24 h at 36 °C.

2.2 Antibiotic sensibility

Identified strains susceptibility to different antibiotics was determined by using the plate diffusion method, so the strains were sowed in Luria Bertani agar (LB), and incubated at 36 °C, during 24 h. Subsequently, colonies were translated to a tube with 5 ml of LB broth, until it was obtained a turbidity of 0.5 of Mc Farland, then with a sterile hyssop, the colonies were sowed in Muller-Hilton agar plates, after 15 minutes, multidisc for Gram (-) of 30 µg of cephalothin (CF), 30 µg chloramphenicol (CL), 30 µg ceftriaxone (CRO), 10 µg ampicillin (AM), 30 µg amikacin (AK), 30 µg trimethoprim (SXT), 30 µg cefotaxime (CTX), netilmicina (NET), 5 µg pefloxacin (PEF), 100 µg carbenicillin (CB) and 30 µg nitrofurantoin (NF), were placed (Sanofi, Mexico), also it was added filter paper discs with same characteristics and impregnated with antibiotics: 30 µg of Kanaminine (K) and tetracycline (T) (SIGMA CHEMICAL).Plates were incubated at 30 °C during 24 h, after this period it was measured the halos of inhibition with a vernier. Strains were classified as Resistant (R), Intermediate (I), or Susceptible (S), depending on the diameter of halos, including discs' diameter (6mm) [10].

2.3 Plasmids extraction

The isolated strains were sown in tubes with 5000 µl of Luria Bertani broth, and incubated in water bath with agitation at 36 °C during 24 h, to begin extracting plasmids by alkaline lysis method [11].

Extractions were treated with bovine pancreatic RNAsa I-AS type at a concentration of .01 µg/ml, and were incubated at water bath at 60 °C during 10 min.

10 µl of the obtained samples were inoculated in the wells of agarose gels at 0.6% for electrophoresis analysis. Electrophoresis was made at a voltage of 70 V and power of 250 W. Gels were dyed during 45 min and revealed with a solution of ethidium bromide dissolved in distilled water at a concentration of 0.5 µg/ml. Once the gels were revealed, it was washed with water during 30 seconds to eliminate excesses of ethidium bromide and were placed in a UV rays transilluminator of short wave. Photographs of gels were taken with an instantaneous Polaroid camera with Polaroid 667 film cartridges.

Extractions from isolated strains, were ran simultaneously in gel with the marker of known molecular weight: GENE RULER TM Ikb DNA LADDER; finally, the relative motility of plasmids and reference marker was measured by applying a reverse rule of three to obtain molecular weights in number of base pairs, in function of number of known base pairs of the marker (10000 pb).

All the procedure was made with collection strains: *Aeromonas hydrophila* (ATCC356), *Aeromonas caviae* (ATCC154), *Vibrio alginolyticus* (ATCC177) and *Vibrio parahaemolyticus* (ATCC178) as positive control [12] statistical treatment was made, for confidence intervals of 95% and 99%, and within and between all phases, to accept or reject the hypothesis : H_{null}: The bacterial load of oyster, *Crassostrea virginica*, does not significantly differs with respect to number of plasmids-R and susceptibility to used antibiotics, during its process of commercialization, since the collection until its distribution, sale and consumption in different centers of Mexico City, Hn: ($\mu_R=\mu_D=\mu_V=\mu_V$), and as investigation hypothesis: H_{Invs}:($\mu_R \neq \mu_D \neq \mu_V \neq \mu_C$).

3. Results

3.1 Collection phase

In this phase it was obtained a total of 87 Plasmids-R, 33 correspond to the sampling zone called Laguna camaronera (LC); 27 in the zone denominated Buen País (BP) and 27 in the Zona Laguna (LG). The weights of plasmids obtained goes from 4163 bp until 13333 bp.

The 79% of plasmids were detected in enterobacteria, with molecular weights of 8000 and 8500 bp, corresponding to different species of *Escherichia* and *Enterobacter,* respectively. The most elevated molecular weights, 13333 bp, are of different species of genus *Klebsiella.* 17% of plasmids were extracted from five different species of genus *Vibrio,* with 10000 bp. Finally, four strains of *A. hydrophila*, only had 4% of plasmids that were of 9500 and 10000 bp. Strains of species *Pseudomonas*, did not have plasmids (Table 1). Obtained calculations by applying unilateral variance analysis [12], to recollected samples in the three sampling zones of Alvarado, Veracruz lagoon, the phase of recollection register: $gl_{b\ (between)=}$ 2 and $gl_{wi(within)=}$ 6, at trust level of 0.05 that must be at least 5.14 and level 0.01, must be equal or higher than 10.92, so calculated and obtained rate F for all possible combinations between data of three samples zones, sampling period, different bacterial groups and molecular weights, the alternative was to accept Hn: ($\mu_{LC}= \mu_P = \mu_{Lg}$).

Table 1: Presence of Plasmids-R, in isolated bacteria of *C. virginica*, collected at Alvarado, Veracruz lagoon.

	Collection phase									
	Tío Luís			**Manglar**			**Arbolillo**			Plasmid (pb)
Bacteria	**Ja- Ap**	**Ma- Jul**	**Au-De**	**Ja- Ap**	**Ma- Jul**	**Au- De**	**Ja- Ap**	**Ma- Jul**	**Au- De**	
Enterobacteria	13	4	9	10	2	8	8	8	6	4500- 13330
Vibrio	2	1	3	2	1	2	1	1	2	9500- 10000
Aeromona and *Pseudomona*	0	1	0	1	1	0	0	1	0	8500

75% of isolated strains that had plasmids in this phase, presented resistance to ampicillin and nitrofurantoin, and 85% to carbenicillin. By applying the same statistical analysis to susceptibility response to the different tested antibiotics, obtained calculus in a significant reason, for $gl_{b(between)}$11 and $gl_{wi(within)}$ 36, at trust level of 0.05 that must be at least of 5.14 and at level 0.01 must be equal or higher than 10.92, so calculated and obtained reason F=0.1 lead to accept the Hn: ($\mu_{LC}= \mu_P = \mu_{Lg}$), indicating that bacterial strains are showing resistance to antibiotics: ampicillin, carbenicillin and nitrofurantoin.

3.2 Distribution phase

In this phase it was extracted 37 Plasmids-R with 18 different molecular weights, from 2216 pb up to 15833 pb. 84% of plasmids were extracted from enterobacteria of genus *Escherichia*, *Enterobacter, Salmonella* and *Shigella*, isolated during the sampling period of September-December, at the same time the 29% presented a molecular weight of 9160 pb, 20% of 8750 bp and 1000 bp. Highest molecular weight, 15833 bp, was of *E. coli* II strains. 11% of plasmids that were obtained from bacteria of genus *Vibrio*, registered molecular weights of 2916 bp, 3333 bp and 7500 bp, were obtained from 6 strains of 2 different species: *V. cholerae El Tor* and *V. fluvialis*. As the previous phase, strains of specie *Aeromonas hydrophila*, only had 6% of plasmids, with molecular weights of 7083 bp and 5163 bp (Figure 2). Regarding to susceptibility of the 39 isolated strains in this phase, 35% showed ampicillin resistance and 53% to cephalothin. Unilateral variance analysis [12] registered a gl_{wi}= 6 and gl_b= 2, F=0.5 and F=3.5, lead to accept Hn and ($\mu_1=\mu_2=\mu_3$), finding that there is no significant difference between obtained plasmids quantity in the three different sampling periods, nor at extracted plasmids of different bacterial groups, was not found significant difference between molecular weights of bacteria plasmids in the different sampling periods.

Table 2: Presence of Plasmids-R obtained from isolated bacteria of *C. virginica* during its distribution in Mexico City.

Distribution phase (Mexico City)				
Bacteria	**Jan-Apr**	**May-Jul**	**Aug-Dec**	**Plasmid molecular weight (pb)**
Enterobacterias	7	7	7	2216-19000
Vibrios	1	1 1	1	2916- 10000
Aeromonas and Pseudomonas	1	0	1	2500-10000

3.3 Commercialization phase

In this phase were identified a total of 203 plasmids, 69 in January-April period, 74 in May-July and 60 in the last period of sampling, 76% were extracted from enterobacteria, 14% from *Vibrio* and 10% from *Aeromonas* and *Pseudomonas*. 39 different molecular weights were registered; values were from 2216 bp up to 19000 bp. Most presented molecular weight was of 10000 bp, obtained by species of *Escherichia coli* and *Salmonella*. Susceptibility to 12 antibiotics was measured in 47 strains from different species of the identified groups, from which, 74% registered resistance to carbenicillin, 74% to ampicillin, 70% to cephalothin, 38% to nitrofurantoin and 30% to cefotaxime (Table 3). Variance analysis of obtained samples in this phase, registered a gl_i= 15 and gl_{be}= 2, reason F=0.03, and F=0.006, which lead to accept the Hn: ($\mu_1=\mu_2=\mu_3=\mu_4=\mu_5=\mu_6$), because there was not significant difference between plasmids quantity from isolations of the six different studied locals, that sell oyster in the three analyzed periods, and also there was no significant difference between plasmids of different bacterial groups. Nevertheless, when applying the same analysis to ported plasmids number by the three bacterial groups, observed a significant difference between number of isolated plasmids in enterobacteria, in *Vibrio* and other bacterias (*Aeromonas* and *Pseudomonas*). The enterobacteria ported 72% of obtained plasmids, *Vibrio* 16% and 12% by other bacterial groups. From the 38 strains of different species where susceptibility to antibiotics was measured, it was found that 60% had resistance to cephalothin, 66% to ampicillin and 47% to carbenicillin.

Table 3: Presence of Plasmids-R, extracted from isolated bacteria of *C. virginica* in points of sale in Mexico City.

	Commercialization phase (Mexico City)			
Bacteria	**Jan-Apr**	**May-Jul**	**Aug-Dec**	Plasmids Molecular weight (bp)
Enterobacterias	47	58	52	2216-19000
Vibrios	15	9	6	2916- 10000
Aeromonas and *Pseudomonas*	12	8	2	2500-10000

3.4 Consumption phase

In this phase, it was extracted 192 Plasmids-R, of which 76% were obtained from enterobacteria species, 22% from *Vibrio* species, and 2% from *Aeromonas* and *Pseudomonas*. Likewise, during the months of January-April strains they behaved 43% of the plasmids, in May-July were isolated which obtained 29% from September to December and 28%. 32 different molecular weights were recorded from 2500 bps to 15000 bps.

Of the 38 strains of different species that susceptibility to antibiotics was measured, 60% presented resistance to cephalothin, 66% to ampicillin and 47% to carbenicillin (Table 4). Variance analysis of obtained samples in this phase, showed gl_i= 15 and gl_{be}= 2, reason F= 1.1, F=0.45 and F=0.019 (Table 1), this lead to accept the Hn: ($\mu_1=\mu_2=\mu_3=\mu_4=\mu_5=\mu_6$), because there is not a significant difference between plasmid quantity of the isolations of the six different studied locals that sell oyster during the three analyzed periods, nor between extracted plasmids of the different bacterial groups. In the same way, when applying the same statistical analysis to find difference between 3 sampling periods, withe same trust levels as the previous phases, and gl_i= 6 and gl_{be}= 2, obtained reason F=38, indicated that there is significant difference between total number of isolated Plasmids-R between the different bacterial groups (Table 4).

Table 4: Presence of extracted Plasmids-R of isolated bacteria of *C. virginica* in consumption points.

	Consumption phase (Mexico City)			
Bacteria	**Jan-Apr**	**May-Jul**	**Aug-Dec**	Plasmids Molecular weight (bp)
Enterobacterias	56	41	49	2216-15000
Vibrios	23	15	3	2500- 10000
Aeromonas and *Pseudomonas*	4	0	2	5000-8000

Finally, the comparative variance analysis of number of Plasmids-R, during the complete commercialization process, from recollection to oyster consumption, where only for trust level of P< 0.05, and gl_i 9 and gl_{be} 2, the calculated and obtained reason F= 8.7, F= 6, F= 5.3, F= 0.47 and F= 4.2; lead to reject Hn: ($\mu_R=\mu_D=\mu_V=\mu_C$), and accept H $_{invs}$($\mu_R\neq\mu_D\neq\mu_V\neq\mu_C$). However, for a rust level of P=0.01 obtained reason F lead to

accept Hn. This indicates the existence of significant difference between isolated Plasmid-R quantities of the four different phases.

4. Discussion

Alvarado, Veracruz lagoon in the oyster collection zone, presented lower number of isolated species, of each one of the different bacterial groups, Enterobacteria, *Vibrio, Aeromonas*. There was a decrease in number of species in the distribution phase, except *Vibrio*, that in this phase increased its presence, that might be due to a cross contamination with marine species, by being transported in the same containers when transported to distribution centers for their sale. This was intensified in the phase of commercialization and consumption, by having an increase in the number of isolated species: 21% in sale and 23% in consumption. Differences in species of genus *Vibrio, Aeromonas* and *Pseudomonas*, is more notorious than in non-enteric bacterial groups, possibly because of the treatment of oysters with antibiotics, or by the chlorine supply in the oyster water [14].

The number and species of identified bacteria in the four phases, exceed the specified limits in standard NOM-031-SSA1-1993, although this norm only take into account the serotype O: 1 toxigenic of *V. cholera*, even though there are other bacterial groups of sanitary importance that are of the enterobacteria group, within which are the genus: *Enterobacter*, *Klebsiella*, *Escherichia* and *Citrobacter,* this results match with reported by other authors (14), that mention that the high frequency of pathogen microorganism like *Escherichia coli*, V*ibrio* spp, *Salmonella* spp. and *Shigella* ssp. in oysters, is one of the greatest health issues in the country and mainly in state of Veracruz.

Other authors [15]. mention that enterobacteriaceae *Salmonella enteritidis, S. typhimurium, Shigella flexneri* and *S. dysenteriae,* are organisms associated to reactive arthritis. *Salmonella* also can cause osteomyelitis and septic arthritis. Most of the infected develops oligoarthritis after one or two weeks of being infected by *S. typhimurium* or *S. enteriditis*, mainly affecting, wrists, knees and ankles, being just the two species of *Salmonella* founded in oysters.

Regarding to genus *Aeromona,* it is reported as a causal agent of intestinal infections like acute gastroenteritis in human, mainly in immunologically compromised people [16, 17]. *Pseudomonas aeruginosa* is associated to pneumonias [18]. *Citrobacter* genus is frequently a causal agent of human infections, especially urinary infections, neonatal meningitis, brain abscess, vertebral osteomyelitis and spinal abscess, is one of the most important pathogens in units of hospital neonatal care [19]. Because of all the above, consumption of oyster that comes from lagoon system of Veracruz, commercialized and consumed in Mexico City, is of high risk because these bacteria are pathogens for humans. This is because the bivalve mollusks are particularly prone to transmit pathogenic enteric bacteria because in the waters where they grow and collected, they are exposed to fecal contamination transported by wastewaters [20]. On the other hand, the process of filtration that they make for their alimentation, favors the accumulation of bacteria in their digestive system that are hard to eliminate even during cleaning or depuration processes [13]. Also in this study it is proved that during the collection, distribution, commercialization and consumption process, it can acquire or increase the bacterial contamination due to a wrong sanitary management through the process, which is seriously aggravated against the presence of plasmids-R in all phases of commercialization process, a presence that have a significant increase (87 in recollection point, 37 in distribution, 203 in sale and 192 in consumption) probably due to treatment with antibiotics to which oysters are subjected, which is demonstrated by obtaining that in collection phase, 75% of isolated strains presented resistance to ampicillin and nitrofurantoin, and 85% to carbenicillin; in distribution centers of the product the 35% and 53% of isolated strains presented resistance to ampicillin and cephalothin respectively; in the different sealing points of oyster it was registered strains with resistance to carbenicillin in 74%, 70% to ampicillin, 38% to cephalothin and nitrofurantoin, and 30% to cefotaxime. In consumption phase 74% of the strains presented resistance to carbenicillin, 70% to cephalothin, 38% to nitrofurantoin, and 30% to cefotaxime; possibly in an attempt to lessen the effect of the high load bearing of Enterobacteriaceae. In conclusion, it was observed that 60% of all isolated strains showed resistance to cephalothin, 66% to ampicillin, and 47% to carbenicillin. Observed resistance to antibiotics in the studied strains can be due to the indiscriminate use of antibiotics or deficient sanitary conditions that help the dissemination of microorganism and acquisition of Plasmid-R and transposons that determine dissemination of antibiotic resistance [18, 21].

The public health risk of the elevated presence of pathogenic bacteria for human, in oyster *C. virginica*, is increased by the presence of Plasmids-R, which leads to presence of incontrollable infections and a serious problem to health sector.

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