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Assessment of Halophilic Bacteria in the Salt Flats and Wildlife Refuge in Cabo Rojo, Puerto Rico and their Antibiotic Resistance Pattern

Franco Negrón, Jayleen Duprey, José Roig and Karlo Malavé-Llamas*

School of Science and Technology, Universidad del Este, Puerto Rico

***Corresponding Author**: Karlo Malavé-Llamas, School of Science and Technology, Universidad del Este, Carolina, Puerto Rico.

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Abstract

Antibiotic resistance in clinical and environmental samples is an emerging global threat that endanger more human population every day. Horizontal gene transfer of antibiotic resistance genes is phenomena already known to occur in the environment; however, is less characterized in microbial communities associated with extreme environments such as extreme halophiles. With this research we try to answer the question, if antibiotic resistance is pervasive in extreme environments. The Salterns of Cabo Rojo are composed of an estuary with a diversity of ecosystem with a salt concentration that could reach to 30%. We took five (5) water samples from different salter's ponds with pH ranging from 7.3 to 8.4. Samples were processed and incubate at 39°C on MGM and Hv-YPC for 3 - 5 days. We isolated 802 CFU, 129 CFU and 963 CFU. Three (3) presumptive species of bacteria were identified using the BIOLOG Gen-III System; *Acidovorax facilis, Bacillus cecembensis* and *Virgibacillus necropolis*. The Kirby-Bauer Method was used to assess the isolates antibiotic resistance patterns to 10 antibiotics, which showed resistance to Chloramphenicol, Kanamycin, Gentamicin, Neomycin and Rifampin. Antibiotic resistance on the Cabo Rojo Salterns will indicate anthropogenic impact on the environment.

Keywords: *Halophiles; Puerto Rico; Antibiotic Resistance; Salt Flats; BIOLOG*

Introduction

Salt has been used to flavor and preserve food and even today, continue to practice conservation of cod, cured meat, olives and vegetables in brines. Salt has been present in most human societies for their importance and usefulness in the food industry. Notably, some microorganisms have managed to thrive in environments with high salt concentrations. Today we know that halophilic bacteria require concentrations of salt that ranges from 10% and up to 34% of NaCl. Halophiles, are part of a group of organisms known as extremophiles. Extremophile organisms are adapted to environmental conditions difficult to bear for most living things. The Cabo Rojo Salterns (Figure 1) comprise an area of 1,249 acres of lagoons, salt marshes, mangroves and dry forest. This resource is listed as a category 1 by the US Fish and Wildlife Service. Meaning that the natural value to wildlife is unique and irreplaceable both at national and regional level. The Salterns was the first industry of its kind in Puerto Rico and one of the first in America. The great historical and ecological value of the Salterns makes it a natural heritage of incalculable importance for humanity. In 1999 the Salterns became part of the US Fish and Wildlife Service Refuge at Cabo Rojo. The Cabo Rojo Salterns belong to the geometric province known as South coastal plain in Puerto Rico. The Solar Salters, at Cabo Rojo, originate from the evaporation of sea water due to high temperature and low precipitation; having an ion composition dominated mainly by Na and Cl, making it an extremely halophilic environment. The [NaCl] of these man-made pools range from 3% to 35%. Additionally, this environment has: a low oxygen concentration, high light intensity, ample nutrient availability (eutrophic), and neutral to alkaline pH. Even though the microbial diversity present in this niche, is low compared to mesophilic environments, representatives can be found from the three domains of life: Archaea, Bacteria and Eukarya [1,4]. Like other salterns, the Cabo Rojo system consists of a series of interconnected ponds in which the concentration of salt increases as sea water evaporates because solar radiation and light intensity.

Figure 1*: A crystallizer pond of salt, Cabo Rojo Salterns, Puerto Rico.*

Antibiotic resistance is one of the aspects of increasing attention regarding human health and has been for a while. Increased infections caused by pathogenic bacteria resistant to antibiotics is producing an "arms race", evolutionarily speaking, in which the development of new antibiotics is behind the ability of microorganisms to avoid its effect by increasing resistance to them. The cause of antibiotic resistance is naturally driven, that is, the bacteria themselves have evolved mechanisms to inactivate the antibiotic substances produced as defense element or competitive strategy with other microorganisms [2]. But this resistance, or adaptive strategy of bacteria is enhanced by human activity with the use of antibiotics for the treatment of human and animal diseases. The anthropogenic selective mechanisms increase the likelihood of contact with bacteria antibiotics has led to a rapid selection of bacterial populations, favoring those resistant [3]. Although antibiotic resistance can occur in the absence of antibiotic gene mutation [5], the overuse of antibiotics has led to an alarming increase in resistance [6]. The overuse of antibiotics increased excretion, primarily through wastewater, into the environment resulting in an upsurge proportion of environmental resistant bacteria. Issuing bacteria into the aquatic environment also favors genetic exchange with previously resistant populations, thereby increasing the dispersion of the resistance capacity in the environmental bacteria [5,7,8]. Resistant bacteria get into rivers and other water bodies through wastewater. One aspect of interest is to determine the ability of these bacteria to survive in natural conditions and to exchange genetic material in such environments [5,9]. Antibiotic resistance in clinical and environmental samples is an emerging global threats that endanger more human population every day. Horizontal gene transfer of antibiotic resistance genes is phenomena already known to occur in environmental microbial organisms including marine microbial ecosystems. However, is less characterized in microbial communities associated with extreme environments such as extreme halophiles.

With this research, we try to answer the question, if antibiotic resistance is as pervasive in extreme environments, like hypersaline ecosystem, of closely populated areas such as Cabo Rojo Salterns? The salterns of Cabo Rojo are composed of an estuary with a diversity of ecosystem such as mangroves, seagrass beds, and offshore coral reefs. This, wildlife refuge environment, serves as a habitat for cyanobacteria, algae, brine shrimp (*Artemia salina*) [10]. The water on site have a salt concentration that fluctuate between 5 to 30%. Initially we want to focus on the isolation, identification culture-dependent of hyper-saline bacteria and to characterize the environment [11-14]. The other objectives of the study are (a) to evaluate the water abiotic characteristics, and (b) to assess the antibiotic resistant characteristics of the bacterial isolates.

Materials and Methods

Solution #1 (30% Salt Water Stock Solution)

A 30% salt solution was prepared for the growth mediums, using as a reference the recipe on the halo handbook (NaCl 240g/$MgCl_2$.$6H_20$ 30g/$MgS0_4$.$7H_20$ 35g/KCL 7g/1M Tris-CL, pH 7.5 20 ml) for this process, we add pure warm water to the required volume and dissolve the salts completely using a glass stirring rod or a magnetic stirrer [15]. After the solution was prepared, $CaCl_2$ 2H20 was added slowly from a 1M sterile stock solution using 5 ml of the solution per 1L with an adjusted pH of 7.5 and volume. The solution was sterilized (autoclave) at 101 kPa 15lb for 15 minutes and store at room temperature).

Solution #2 (Yeast Peptone Casamino (YPC) Solution)

For the YPC Preparation pure Yeast Extract/Peptone/Casamino acids/1M KOH media was used. This was prepared with high salt concentration, prepared previously. In addition, $CaCl_2$ was added at the end, just before pouring plates, and some Tris-HCL pH 7.5. The final concentration were 0.5% yeast extract 0.1% and 0.1% casamino acids.

Agar Mediums

The enriched agar media was prepared combining the previous solutions with Difco-Bacto Agar A) Combine in a 500 ml duran bottle Pure H20 100 ml/30% SW 200 ml Agar 5g. B) Put a stir bar in the bottle and microwave until agar has dissolved. It tends to heat at the sides, so shake bottles between heating-the solution should become clear, and without particles of agar. Microwave for 10 minutes on high, 10 minutes on medium and twice for 5 minutes on medium. C) Add YPC nutrients (this prevent the yeast extract from being caramelized during microwaving). To each 300 ml bottle batch of YPC add 10X YPC 30 ml and autoclave as soon as possible. After autoclaving, allow to cool to about 57°C, and slowly add 2 ml of 0.5 M $CaCl_2$, to each bottle [15].

Sampling

To make this study possible we took (5) five water samples, with sterile water sample bottles (600 ml), from (3) three different salter's ponds within the Cabo Rojo Salters, at depths of 12 inches below de surface of the pond; with pH ranging from 7.3 to 8.4 and an average temperature of 39°C. The Membrane Filtration Method was used to concentrate the bacteria making possible its isolation. The filters were incubated, for 5 - 7 days, at 39°C on two (2) growth media (i) Modified Growth Medium (MGM) for Haloarchaea with 20%, 25% and 30% of total salt concentration; and (ii) Yeast Peptone Casamino Acids Media (Hv-YPC) with 20%, 25% 30% salt concentration. Subsequently, colonies were counted and phenotypically described and some were selected for further analysis. Isolates samples were culture in MGM Agar, MGM broth and YPC agar. Later on, 2 (two) colonies, of every sample, were re-culture and isolate in fresh MGM agar at different salt concentration (20%, 25%, 30%).

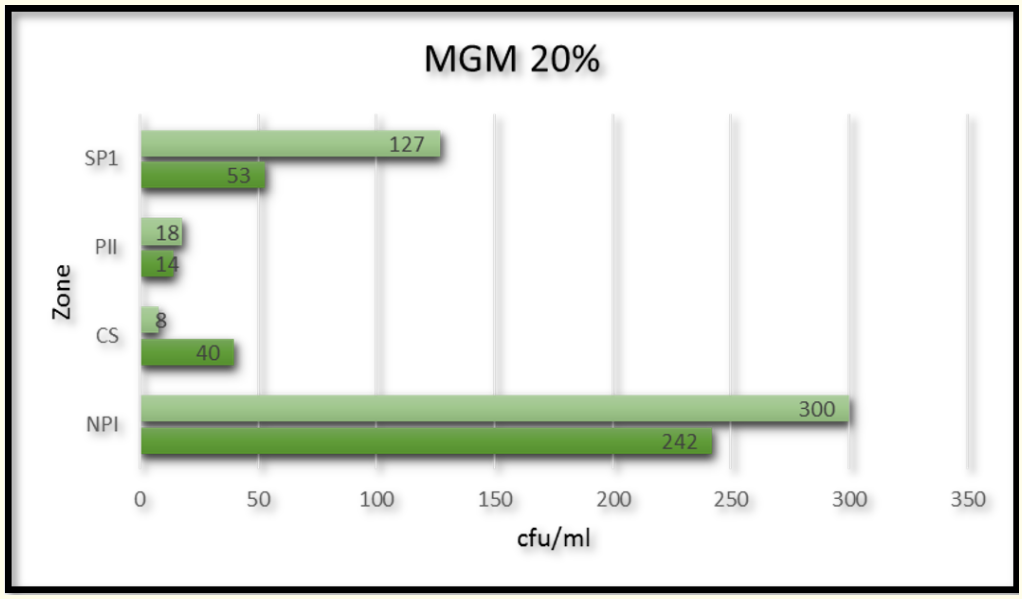
Antibiotic Susceptibility Test

The Kirby-Bauer Method was used to assess the isolates antibiotic resistance patterns to 10 antibiotics: Rifampin 5 µm, Kanamycin 30 µm, Chloramphenicol 30 µm, Neomycin 30 µm, Novobiocin 5 µm, Vancomycin 10 µm, Streptomycin 10 µm, Sulfamethoxazole trimethoprim 250 µm, Gentamicin 10 µm and Trimethoprim 10 µm.

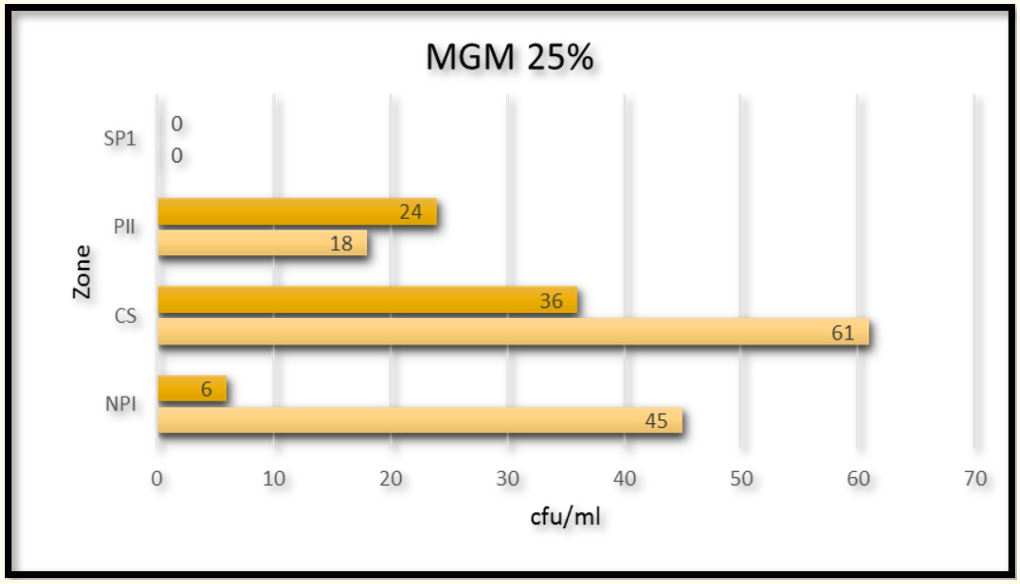
Results and Discussion

We isolated 802 CFU, 129 CFU and 1050 CFU at 20, 25 and 30% of Salt (Figure 2). Three (3) presumptive species of bacteria were identified using the BIOLOG Gen-III System; *Acidovorax facilis, Bacillus cecembensis* and *Virgibacillus necropolis*. Isolates showed growth in the MGM 20%, 25% and 30% of salt; especially on the latter. Diverse profiles of pigments were observed on the prepared agar (Figure 3). Color of pigments produced on agar media were recorded as different tones of pink documented per standard color chart as: Salmon, Watermelon, Baby Pink, Rose Pink and Pink Lemonade. All isolates showed similar growth pattern; circular, rounded and punctiform.

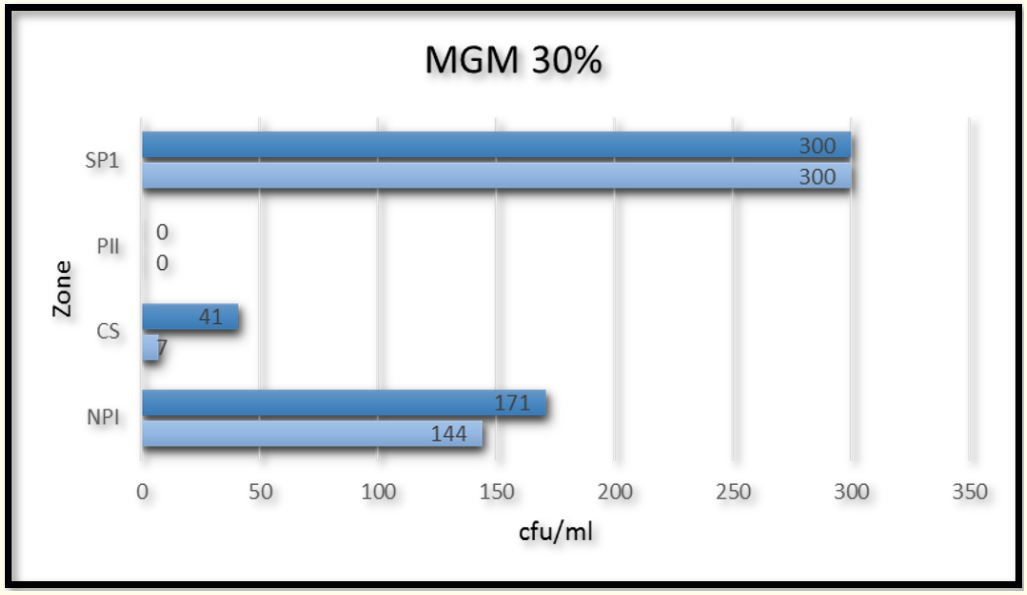
Bacterial Count and Isolation



(a)



(b)



(c)

Figure 2*: This is a figure, represent the isolated CFU by sampling site and salt concentration: (a) A total of 802 CFU were isolated at 20% of salt concentration at the sampling site; (b) A total of 129 CFU were isolated at 25% of salt concentration at the sampling site; (c) A total of 1050 CFU were isolated at 30% of salt concentration at the sampling site.*

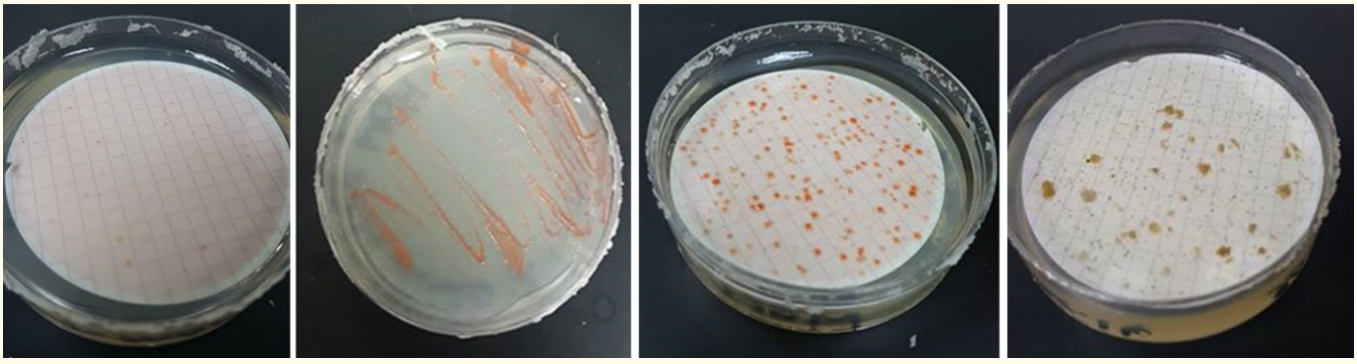


Figure 3*: This is a figure, represent the isolated CFU and their phenotypic characteristics. The pinkish pigmentation of the isolates is evident.*

Antibiotic Resistance

The Kirby-Bauer Method was used to assess the isolates antibiotic resistance patterns to 10 antibiotics: Rifampin 5 µm, Kanamycin 30 µm, Chloramphenicol 30 µm, Neomycin 30 µm, Novobiocin 5 µm, Vancomycin 10 µm, Streptomycin 10 µm, Sulfamethoxazole trimethoprim 250 µm, Gentamicin 10 µm and Trimethoprim 10 µm (Table 1). Although antibiotic resistance is widely spread on the environment it has not been studied on extreme environments frequently. Similar, but not equal patterns had been observed on other hypersaline

environments around the world. The antibiotic resistance patterns observed in this study illustrate the presence of antibiotic resistance genes and mechanisms in nature. Because these organisms were isolated from the environment and have it is important to understand if these genes evolved over time to serve other purposes of bacterial defense or are due to pollution or illegal dumping of the substances.

Antibiotic Susceptibility Test Kirby-Bauer											
ID Sample (%MGM)	**SXT**	**NB**	**CN**	**TMP**		**RA**	**S**	**K**	**VA**	**N**	**% Resistance**
CS2- MGM 20%	2.9 mm	3.5 mm	0.0 mm	3.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.7 mm	0.0 mm	60%
IIP2- MGM 20%	2.8 mm	3.1 mm	0.0 mm	3.2 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.7 mm	0.0 mm	60%
PII.2- MGM 20%	2.2 mm	2.8 mm	0.0 mm	3.5 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	70%
CS1- MGM 25%	3.4 mm	2.8 mm	0.0 mm	2.9 mm	1.5 mm	1.0 mm	0.9 mm	2.1 mm	0.0 mm	0.0 mm	30%
P2- MGM 25%	3.2 mm	3.1 mm	0.0 mm	3.5 mm	0.0 mm	0.0 mm	0.9 mm	0.0 mm	0.0 mm	0.0 mm	60%
CS2- MGM 25%	3.0 mm	2 .8 mm	0.3 mm	3.2 mm	0.0 mm	0.0 mm	1.1 mm	0.0 mm	0.0 mm	0.5 mm	40%
SP1- MGM 30%	0.0 mm	2.1 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	90%
CS3- MGM 30%	0.0 mm	2.5 mm	0.0 mm	0.0 mm	0.3 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	0.0 mm	80%
NP12- MGM 30%	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	N/A

Table 1: *Antibiotic susceptibility test and resistance percentage.*

The process to make the salt water it's a long and tedious one, we select these two mediums because they are specifically for halophiles and includes all the ingredients we need for this microorganism to grow. These two mediums were made at different concentration. MGM at 20%, 25% and 30% and YPC just in 30%. We select these mediums at this salt concentration because we want to work with the extreme halophiles [15]. After this process, was complete and all the mediums were made, we process to find information and characteristics of this salter to help us know more about this extreme halophile and how they can survive in this extreme environment. Yeast extract serve well for the growth of most halophilic bacteria, but all constituents of complex media not. Some lots of peptone proved unsuitable for growth of different kind of halophilic bacteria and could cause the cell to lyse. This is due to the bile salts contained in the peptone, compounds to which halophilic bacteria are sensitive. Other brands of peptone lower the amounts of bile salts and are suitable for the growth of halophilic bacterial.

We identified this samples on the BIOLOG GEN III System. After the 7 days of isolate, we prosecute to identify this microorganism. On this process we fail to identify some samples, we just could identify 3 of the samples because. The biology system it's more for environmental area, and it just can identify halophiles in a rate of 3% to 15%. This system consists in a variety of biochemist process made to the samples and the system tell you the percentage of probability of what kind of microorganism is. Three putative species *Acidovorax facilis*, *Bacillus cecembensis* and *Virgibacillus necropolis* were identified with over 95% of confidence based on metabolic profile and data set of BIOLOG Gen-III System.

Conclusion

Cabo Rojo Salters water on this site have a salt concentration that fluctuate between 5% to 35% (NaCl) for this reason our results on CFU were 802 CFU, 129 CFU and 1050 CFU at 20%, 25%, 30%. It's important to recognize that the season we went to take the samples

was the salter dry season. For this motive, we have this high number of colonies. Using the Kirby Bauer method. Due to extreme environment where they found, no or few resistances against antibiotics was expected, however high resistance was found [16,17]. The antibiotic resistance shows high adaptability and possible horizontal transfer resistance genes from others species. Antibiotic resistance is known to be present in highly populated area and their surroundings, raising the possibility that salter's ponds in Puerto Rico received waters with microbial species with antibiotic resistance genes [18]. Horizontal gene transfer (HGT) is an important aspect of microbial evolution, more common in some organisms than others, and is poorly understood. Occurrence of HGT it is dependent on the incorporation of the transferred DNA into the genome after uptake. Therefore, HGT is more common among closely related organisms. HGT is the primary mechanism for the spread of antibiotic resistance in bacteria, and plays an important role in the evolution of bacteria that can degrade xenobiotic compounds. It is also postulated that HGT promotes the maintenance of a universal life biochemistry and, subsequently, the universality of the genetic code. Although, this is also true for Halophiles; this group of organisms had shown that they have high affinity for HGT with None-Halophiles [19]. The presence of genes resistance to antibiotics emphasized the extent of the environmental horizontal transfer of gene includes extremes environments. These putative gene transfer can alter the microbial ecology of extreme environments such as salter ponds. Therefore, it appears that horizontal gene transfer it is the logical explanation to be responsible for the emergence of antibiotic resistance of the isolates on this study.

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Conflict of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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