

Abanico Veterinario. January-December 2021; 11:1-13. <http://dx.doi.org/10.21929/abavet2021.19>
Original Article. Received: 13/12/2020. Accepted: 29/03/2021. Published: 12/04/2021. Code: e2020-101.

Biochemical identification of potentially pathogenic and zoonotic bacteria in black turtles (*Chelonia mydas*) from the Mexican Pacific

Identificación bioquímica de bacterias potencialmente patógenas y zoonóticas en las tortugas negras (*Chelonia mydas*) del Pacífico Mexicano

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ABSTRACT

Sea turtles naturally have gastrointestinal microbiota; however, opportunistic behavior and pathogenicity of some bacteria have also been reported. Therefore, it is important to generate information on possible risks to turtles and human health. Five monthly field monitoring were carried out with captures of *Chelonia mydas* in the Ojo de Liebre lagoon complex. Physical examinations were performed and their morphometries were recorded; oral and cloacal swabs were made and sowing in McConkey and TCBS culture media. Bacterial agents were isolated and identified using the API®20E system. Turtles' body condition index and percentages of abundance and prevalence of each microorganism were calculated. Finally, the ratio between turtle s' size and the presence of microorganisms was determined. Many *Chelonia mydas* (178) were captured, 523 isolates of gram-negative Enterobacteria from seven different species were obtained; the presence of proteobacteria in *Chelonia mydas* was not related to their age class. Among the microorganisms found, *Vibrio fluvialis* and *Burkholderia cepacia* are zoonotic. These studies allow us understanding the role of microorganisms in diseases of wild populations and risks to public health associated with their illegal consumption.

Keywords: bacteriology, microbiota, marine turtles, potential health threats, pathogens, zoonoses.

RESUMEN

Las tortugas marinas poseen naturalmente microbiota gastrointestinal, sin embargo, también se ha reportado el comportamiento oportunista y patogenicidad de algunas bacterias en estas especies. Por lo tanto, es importante generar información sobre los posibles riesgos para las tortugas y la salud humana. Se realizaron cinco monitoreos mensuales con capturas de *Chelonia mydas* en el complejo lagunar Ojo de Liebre. Se les practicaron exámenes físicos y se registraron sus morfometrías y se calculó su índice de condición corporal; se realizaron hisopados orales y cloacales que se sembraron en medios McConkey y TCBS. Los agentes bacterianos se aislaron e identificaron mediante el sistema API®20E. Se calcularon los porcentajes de abundancia y prevalencia de cada microorganismo. Finalmente, se determinó la relación entre la talla de las tortugas y la presencia de los microorganismos. Se capturaron 178 *Chelonia mydas*, se obtuvieron 523 aislamientos de enterobacterias gramnegativas de siete especies diferentes; la presencia de proteobacterias en *Chelonia mydas* no se relacionó con su clase de edad. Dentro de los microorganismos encontrados, *Vibrio fluvialis* y *Burkholderia cepacia* son zoonóticos. Estos estudios permiten comprender el papel de los microorganismos en las enfermedades de las poblaciones silvestres y los riesgos para la salud pública asociados a su consumo ilegal.

Palabras clave: bacteriología, microbiota, tortugas marinas, amenazas potenciales para la salud, patógenos, zoonosis.

INTRODUCTION

The green turtle of the Eastern Pacific (*Chelonia Mydas*) in danger of extinction, locally known as black/brown turtle, is distributed along the American Pacific coast, occupying different geographical regions during each of its life cycle stages (Cliffon *et al.*, 1982). These agencies are susceptible to specific threats of their environment and of anthropogenic origin (Aguirre *et al.*, 2006), therefore, the study of health conditions of populations in free life, including the presence of infectious agents, is receiving increasing attention for conservation.

Sea turtles in free life naturally house a wide variety of bacteria in their gastrointestinal tract. However, its opportunistic behavior and pathogenicity (Ahasan *et al.*, 2018) have also been reported. In an organism, the communities of associated microorganisms are essential for a wide variety of functions. The development of the host and its health state depend on the presence of an intact microbial community, which plays an important role in all living organisms (Bloodgood *et al.*, 2020). In different species, it has been shown that microbiota promotes the development of organs and tissues, the production of vitamins and essential amino acids that affect the use of fats (Koropatnick *et al.*, 2004), and the response of glucose and lymphocytes to the Intestinal injuries (Warwick *et al.*, 2013). The different bacteria of the gastrointestinal tract of sea turtles can provide various functions that are still unknown and suggest playing an important role in the assimilation of food and their use (Ahasan *et al.*, 2017). However, these microorganisms can also cause host damage, for example, increasing susceptibility to intestinal inflammations and infectious diseases mainly (Garner *et al.*, 1995). Internationally, the presence of various bacteria in sea turtles has been reported such as: *Salmonella*, *Mycobacterium*, *Escherichia Coli*, *Citrobacter Freundii*, *Edwardsiella* sp., *Vibrio Alginolyticus*, *Vibrio Cholerae*, *Vibrio Fluvialis*, *Vibrio Furnisii*, *Vibrio parahaemolyticus*, *Aeromonas* and *Proteus*, between others (Work *et al.*, 2003; Orós *et al.*, 2005; Santoro *et al.*, 2006; Zavala-Norzagaray *et al.*, 2015). They have been identified as opportunists and potentially pathogenic for turtles. In addition, some adverse health effects have been reported in humans that consume meat and eggs of sea turtles infected with zoonotic pathogens, a common illegal practice in coastal countries around the world (Aguirre *et al.*, 2006). In Mexico, the information about these microorganisms related to diseases in sea turtles is still limited. Therefore, it is important to generate truthful information about the possible risks for turtles and human health associated with its illegal consumption. This study aimed to provide the baseline on potentially pathogenic bacterial agents for turtles and zoonotic type, through bacteriological crops and biochemical methods in black turtle samples (*C. Mydas*) in free life of Ojo de Liebre lagoon complex, Baja California Sur (BCS), Mexico.

MATERIAL AND METHODS

Study site

The Ojo de Liebre Lagoon (LOL) and Guerrero Negro Lagoon (GNO) belong to the Ojo de Liebre lagoon complex and they are part of the Biosphere Reserve "El Vizcaíno", located in the North Pacific of BCS, between latitude 27° 35' and 28° 15' north, and longitude 113° 50' and 114° 20' west. Both lagoons are hypersaline and do not have fresh water contributions. They have a high degree of evaporation and a slow water circulation. Overall, the oceanographic and climatological characteristics of the region provide a high-wealth habitat, which represents one of the main feeding sites and development of black turtles in their youth and adult stages in the Mexican Pacific ([Reséndiz et al., 2018a](#)).

Collection and processing of samples

Five monthly field outputs since September 2019 were performed, 2020, with captures of black turtles in LOL and GNO. All catches were performed with monofilament networks type "castle" during the day. The captured animals were subjected to a physical examination ([Norton, 2005](#)), and then oral and cloacal scrapes were performed with sterile swabs. For this, circular and rotating movements were performed with the swab on the internal surface of the mouth and the internal surface of the cloaca and they were placed in culture medium Stuart Copan® for transport. Next, the carapace curved length (CCL, centimeters) was recorded, the carapace straight length (CSL, centimeters) and the weight (Kilograms) ([Bolten, 1999](#)). Adults were considered adults with CCL higher than 77.5 cm ([Márquez, 1996](#)). Immediately afterwards, the turtles were marked with Inconel 625 metal plates on the rear fins ([Balazs, 1999](#)) and released unscathed at the capture site. The samples were refrigerated at 4 °C and they were transferred to the microbiology laboratory of the Autonomous University of South California. They were sown in McConkey agar for the identification of enterobacteria and gram-negative bacilli (incubation for 24h at 37 °C), and in agar thiosulfate citrate bilis sucrose (TCBS) for the bacteria identification of the genus *Vibrio* (incubation for 24h at 30 °C). Subsequently, the colonies were isolated and processed with the biochemical testing system for the identification of bacteria of the Enterobacteriaceae family and other API®20E bacilli. The reactions were read according to the identification table "API®20E analytical profile index".

Analysis of data

The body condition index (BCI) of each body was calculated with the formula proposed by [Bjorndal et al., \(2000\)](#):

$$BCI = \frac{Weight * 10000}{CSL^3}$$

Additionally, the standard means and standard deviations of CCL, CSL, weight and BCI were calculated. The percentages of relative abundance of each bacterial agent in the mouth and cloaca with the following formula were estimated:

$$\text{Relative abundance (\%)} = \frac{N^{\circ} \cdot \text{Isolations of a species}}{\text{Total isolates}} * 100$$

Next, the prevalence of each agent was calculated in the organisms with the formula:

$$\text{Prevalence (\%)} = \frac{N^{\circ} \text{ affected individuals}}{N^{\circ} \text{ Individuals in a population}} * 100$$

Finally, the Kruskal-Wallis test was used to determine the relationship between the size of the organisms (CCL) and the presence of the different species of bacteria. The values of $P \leq 0.05$ were considered statistically significant. Statistical analyzes were performed in R version 3.6.2.

RESULTS

Many black turtles (178) were captured, which measured 72.37 ± 11.98 cm of CCL, 67.27 ± 11.26 cm CSL and weighed 44.61 ± 21.15 kg. Turtles were classified as 125 juveniles and 53 adults and their BCI was 1.38 ± 0.18 . 523 isolates associated with gramnegative enterobacteria of seven different species (Table 1) were obtained.

In the mouth of turtles, the most abundant bacteria were *Kleibsiella* sp. (31.56%), followed by *Citrobacter Freundii* (29.79%), *Enterobacter* sp. (23.01%), *Pseudomonas aeruginosa* (15.34%) and *Burkholderia cepacia* (0.29%), while in the cloaca the most abundant was *Proteus* sp. (42.93%), *Citrobacter Freundii* (28.80%), *Aeruginosa Pseudomonas* (19.57%), *Vibrio Fluvialis* (4.89%) and finally *Burkholderia cepacia* (3.80%) (Figure 1).

The Kruskal-Wallis test showed no significant differences between the presence of the different microorganisms and the CCL of turtles (Ji-square = 5.75, gl = 6, p = 0.45 value) (Figure 2).

Table 1. Quantitative description of the isolated bacterial agents of black turtles (*Chelonia Mydas*) youth and adults at Ojo de Liebre lagoon complex, Baja California Sur, Mexico.

Family	Species	Total isolates	Relative abundance (%)	Prevalence (%)	Isolates in mouth	Relative abundance in mouth (%)	Isolations in cloaca	Relative abundance in cloaca (%)
Enterobacteriaceae	<i>Citrobacter freundii</i>	154	29.45	86.52	101	29.79	53	28.80
	<i>Klebsiella sp.</i>	107	20.46	60.11	107	31.56	0	0.00
	<i>Enterobacter sp.</i>	78	14.91	43.82	78	23.01	0	0.00
Pseudomonadaceae	<i>Pseudomonas aeruginosa</i>	88	16.83	49.44	52	15.34	36	19.57
Morganellaceae	<i>Proteus sp.</i>	79	15.11	44.38	0	0.00	79	42.93
Burkholderiaceae	<i>Burkholderia cepacia</i>	8	1.53	4.49	1	0.29	7	3.80
Vibrionaceae	<i>Vibrio fluvialis</i>	9	1.72	5.06	0	0.00	9	4.89
Total		523	100		339	100	184	100

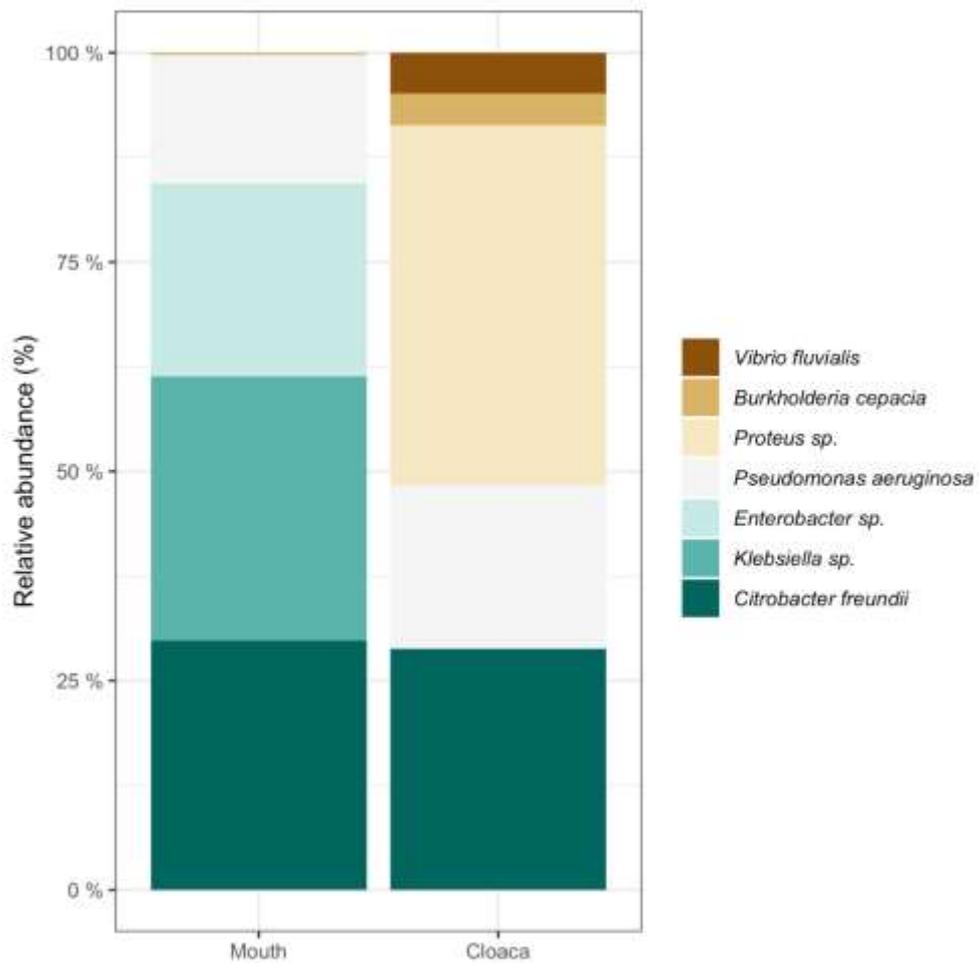


Figure 1. Relative abundance of bacterial agents in the mouth and cloaca of black turtles (*Chelonia Mydas*) at the Ojo de Liebre lagoon complex, Baja California Sur, Mexico

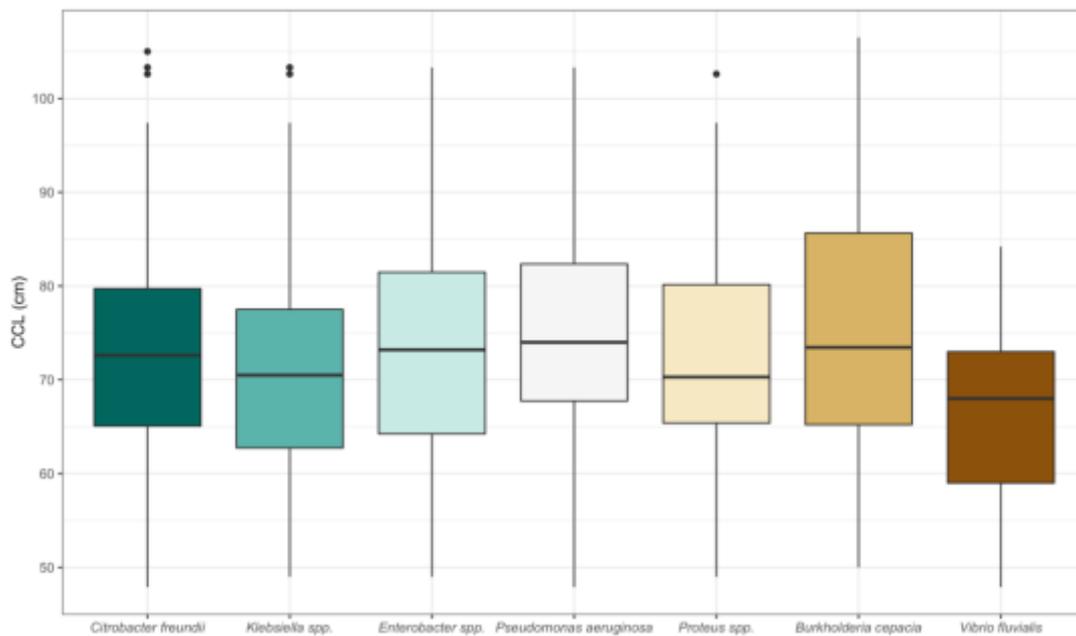


Figure 2. Bacterial agents present in the different carapace curved length (CCL) of black turtles (*Chelonia Mydas*) in Ojo de Liebre lagoon complex, Baja California Sur, Mexico

DISCUSSION

Turtles were classified as juveniles and adults according to the size reported for the area (Márquez, 1996). Its BCI coincided with the previously reported for the area (Reséndiz *et al.*, 2018b) indicating that turtles had good nutritional status and presumably the capacity for favorable reproductive performance. The physical examination showed no evidence of clinical signs, serious injury or diseases that compromised the functioning of turtle organs and systems, nor that they put their normal activities at risk or attempt against their integrity (Norton, 2005).

It is important to consider that the differences in the microorganism communities of sea turtles are attributed mainly to ecological and ontogenic factors (trophic level, diet, habitat, etc.) (Ahasan *et al.*, 2018), so the gastrointestinal bacterial composition differs significantly between populations in free life and rehabilitation agencies (before hospitalization and after rehabilitation) (Pace *et al.*, 2019; Bloodgood *et al.*, 2020). In general, the high prevalence of proteobacteria has been associated with dysbiosis as well as deterioration in the health status of sea turtles (Ahasan *et al.*, 2018). *Citrobacter Freundii* showed the highest prevalence (86.52%). This gramnegative bacteria of the Enterobacteriaceae family has been reported earlier in sea turtles internationally (Santoro *et al.*, 2006), nationally in black turtles in Sinaloa (Zavala-Norzagaray *et al.*, 2015) and in yellow turtles (*Caretta Caretta*) in the Gulf of Ulloa, BCS (Reséndiz *et al.*, 2019). It is considered an opportunistic pathogen associated with infections in juvenile animals and secondary infections in immunosuppressed animals (Glazebrook and Campbell, 1990), and is not zoonotic (Johnson-Delaney, 2014). In sea turtles, the infectious dose and the incubation period are unknown. It has been reported that its transmission is fecal-oral by ingesting contaminated foods or by direct contact between the carrier and susceptible and immunosuppressed organisms (Johnson-Delaney, 2014), this species is found in the gastrointestinal tract of animals and uses bodies of water as a reservoir (Work *et al.*, 2003). *Klebsiella* sp. had a prevalence of 60.11%. This gramnegative bacteria of the Enterobacteriaceae family was previously reported in sea turtles internationally (Warwick *et al.*, 2013) and national in yellow turtles in the Gulf of Ulloa, BCS (Reséndiz *et al.*, 2019). This pathogen plays an important role as a cause of opportunistic infectious diseases, mainly in immunosuppressed organisms and frequently in juvenile animals (Work *et al.*, 2003). It has also been associated with bacteremia, injury, respiratory and urinary infections (Johnson-Delaney, 2014). The infectious dose and the incubation period in sea turtles are unknown. It is not zoonotic and their vectors are unknown, although it has been reported that its most frequent transmission path is through the feces (Jacobson, 2007). This genre can be isolated from the soil, bodies of water, skin, nasopharyngeal exudates, or of the gastrointestinal tract of carrier turtles (Glazebrook and Campbell, 1990) and can survive in marine environments for several hours or in clinically healthy animals for long periods (Tan *et al.*, 2009). *Pseudomonas aeruginosa* presented a prevalence of 49.44%. These gram-negative bacilli belong to the Pseudomonadaceae family (Buller, 2004) and have been studied in sea turtles at an international level (Work *et al.*, 2003; Santoro *et al.*, 2006) and national (Reséndiz *et al.*, 2019). These are opportunistic pathogens suggest an

increased risk of disease in immunosuppressed and fibropylomatosis juvenile turtles ([Work et al., 2003](#)), most of its conditions arise from the colonization of the respiratory and urinary tract or infections of deep dissemination that can cause pneumonia, bacteremia and chronic respiratory infections ([Buller, 2004](#); [Jorgensen and Ferraro, 2009](#)). They are not zoonotic and in sea turtles, vectors are not known ([Work et al., 2003](#); [Johnson-Delaney, 2014](#)). The infectious dose in sea turtles is also unknown and its incubation period varies according to the infection ([Orós et al., 2005](#); [Jorgensen and Ferraro, 2009](#)). This species is transmitted by direct contact with contaminated water, aerosols or aspiration by contact of mucous membranes with discharges of infected conjunctivas or upper respiratory tract of infected organisms ([Johnson-Delaney, 2014](#)) and can survive for several months in the marine environment with basic nutrients ([Jacobson, 2007](#)). *Proteus* sp. had a prevalence of 44.38%. These gram-negative bacilli Morganellaceae have been reported in other healthy and sick sea turtle species at the international level ([Santoro et al., 2006](#)), in Mexico, in black turtles ([Zavala-Norzagaray et al., 2015](#)) and in yellow turtles clinically healthy ([Reséndiz et al., 2019](#)). They are considered part of the gastrointestinal microbiota of sea turtles; however, they can generate chronic urinary tract infections such as bacteremia, pneumonia and focal injuries in weakened and emaciated organisms ([Work et al., 2003](#)). They are not zoonotic and no transmission vectors are known. In addition, in sea turtles, the infectious dose is unknown and the incubation period is not well established ([Johnson-Delaney, 2014](#)), although it has been reported that it causes infections when leaving the gastrointestinal tract ([Jorgensen and Ferraro, 2009](#)). This genus is not transmitted by direct contact between organisms and can be found in bodies of water ([Jacobson, 2007](#)). Survive easily outside the host, especially in areas where there is an animal protein decomposition ([Buller, 2004](#); [Jacobson, 2007](#)). *Enteropose* sp. showed a prevalence of 43.82%. These gramnegative enterobacteria was reported in yellow turtles in the Gulf of Ulloa, BCS ([Reséndiz et al., 2019](#)) and in other marine turtle species at an international level ([Work et al., 2003](#); [Zavala-Norzagaray et al., 2015](#)). It is considered an opportunistic pathogen that has been associated with infectious outbreaks such as fibropylomatosis in juvenile turtles ([Work et al., 2003](#)). It can cause numerous infections, such as pneumonia, sepsis in the intestinal tract and in the urinary tract, which can cause bacteremia ([Work et al., 2003](#); [Orós et al., 2005](#)). It is not zoonotic and do not know each other ([Glazebrook and Campbell, 1990](#); [Warwick et al., 2013](#)). In addition, its incubation and infectious dose period in sea turtles is unknown. However, it is known that it is transmitted by direct or indirect contact of mucous surfaces with the infectious agent and can be transmitted by fecal-oral ([Johnson-Delaney, 2014](#)). This genus is colonizer of the lower gastrointestinal tract of humans, animals and it can often be found in plants, soil, water bodies and is able to survive with a minimum source of energy ([Pace et al., 2019](#)). *Vibrio Fluvialis* had a prevalence of 5.06%. This gramnegative bacillus of the Vibrionaceae family has been reported in sick and healthy sea turtles at an international and national level ([Zavala-Norzagaray et al., 2015](#); [Reséndiz et al., 2019](#)). It is considered an emerging pathogen that is transmitted by eating contaminated foods ([Ahasan et al., 2017](#); [Franco-Monsreal et al., 2014](#)). All its consequences are unknown

in sea turtles, but in mammals cause diarrhea similar to anger, cutaneous infections associated with exposure to aquatic environments and even sepsis in immunosuppressed individuals (Franco-Monsreal *et al.*, 2014). Its period of incubation and infectious dose in sea turtles is unknown. This microorganism is zoonotic (Johnson-Delaney, 2014) and can survive for long periods in the marine environment, where it is widely distributed (Igbinsosa and Okoh, 2010; Franco-Monsreal *et al.*, 2014).

Some reports indicate that it has also been isolated from sewage, animal and human stool, as well as seafood, mainly in *Bivalvos Molluscs* (Alton *et al.*, 2006; Igbinsosa and Okoh, 2010). Finally, *Burkholderia Cepacia* had a prevalence of 4.49%. This gram-negative bacteria of the Burkholderiaceae family has been previously reported at an international and national level in Tortugas Golinas (*Lepidochelys Olivacea*) (Santoro *et al.*, 2006; Zavala-Norzagaray *et al.*, 2015), green turtles (*C. Mydas*) with and without fibropylomatosis (Work *et al.*, 2003) and in clinically healthy yellow turtles (Reséndiz *et al.*, 2019). This pathogenic microorganism can cause chronic pulmonary disease with mucopurulent secretion, characterized by multiple abscesses in the skin and subcutaneous tissues, or a severe sepsis with death in about 7-10 days (Orós *et al.*, 2005; Jacobson, 2007). In sea turtles, the infectious dose is unknown, its incubation period varies from 1 to 14 days (Johnson-Delaney, 2014) and it is considered zoonotic by direct or indirect contact of the mucosa with discharges of injury from infected animals (Warwick *et al.*, 2013; Johnson-Delaney, 2014). It has been reported in mammals, reptiles and fish, considering humans as accidental guests (Buller, 2004; Jorgensen and Ferraro, 2009) It can be found on the ground, bodies of water and areas close to agricultural activities, where it can survive for long periods at room temperature (Buller, 2004; Jacobson, 2007)

The presence of these bacterial agents in sea turtles was not related to their carving; The Kruskal-Wallis test showed no significant differences between the presence of the different microorganisms and the CCL of turtles, which indicates that the found bacteria presented interchangeably in juvenile animals and adults. Within the proteobacteria found in black turtles, *Vibrio Fluvialis* and *Burkholderia cepacia* are zoonotic pathogens, and although they have a low prevalence, they must be monitored regularly to prevent risks in turtles and public health. Since, despite the federal prohibition of capture, consumption and trade in sea turtles in Mexico since 1990, these organizations continue to be captured and consumed. This fact represents a great potential danger for human health (Aguirre *et al.*, 2006), being able to cause extreme dehydration, vomiting, diarrhea and even death to consumers due to the presence of these microorganisms (Alton *et al.*, 2006), in addition to viruses, parasites or pollutants in sea turtles (Zavala-Norzagaray *et al.*, 2015). The information generated warns about possible risks to the health of sea turtles consumers. In addition to being an illegal practice, it is potentially dangerous for public health and affects the populations of the different protected species. This study complements the health assessments of black

turtles in the area and management and conservation plans of agencies and their ecosystems in the Biosphere Reserve "El Vizcaíno", along with local authorities.

CONCLUSIONS

Seven potentially pathogenic bacterial agents were reported for sea turtles in apparently healthy black turtle individuals, of which two are zoonotic. Strong clinical evidence is required to define whether these microorganisms cause diseases and studies that are more specific are needed to clarify the differences between the microbiota and the pathobiota of sea turtles, especially with molecular methods.

Acknowledgment

The authors thank to Everardo Mariano, Oscar Salazar, Noah López, Gabriel Zaragoza and Rafael Buelna Degree from the Biosphere Reserve "El Vizcaíno" of the National Commission of Protected Natural Areas. To Aaron Sánchez, Fabián Castillo, Joaquín Rivera and Antonio Zaragoza of the Environmental Conservation Area and Integral Management and Planning of the Export Company of Salt S.A for your assistance during field work, support, logistics and guidance during the development of this research. Thanks to Carlos Gamboa, Ibrahí Rodríguez and Indira Alejandra Macías Guerrero from the Autonomous University of Baja California Sur (UABCS). This research was carried out under the conditions of the permissions: Office No. SGPA/DGVS/013214/18 and Office No. SGPA/DGVS/12688/19, and all applicable international, national and institutional guidelines for care and use were followed of animals.

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