Fatal *Photobacterium damselae*-induced enteritis in a leatherback turtle (*Dermochelys coriacea*)

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Running page head: PDD enteritis in leatherback turtle

ABSTRACT: Stranded leatherback turtle (*Dermochelys coriacea*) complete pathology reports are rare and the cause of mortality is difficult to determine in many cases. We conducted a complete pathological study of a stranded leatherback turtle from the western Mediterranean. The main finding was a fibrino-necrotizing enteritis with associated bacteria which were identified as *Photobacterium damselae* subsp. *damselae* according to their biochemical and phenotypical characteristics. This report provides evidence of the pathogenic effect of this bacterium in wild sea turtles.

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1. INTRODUCTION

Leatherback turtle (*Dermochelys coriacea*, Vandelli, 1761) is a worldwide distributed sea turtle species that can be seen occasionally in the Mediterranean Sea. Despite their occurrence being much lower than that of loggerhead turtle (*Caretta caretta*) and green turtle (*Chelonia mydas*) (Gómez de Segura et al. 2006), records of this species are not infrequent. Over a period of 20 years, there were 411 reported leatherback turtle strandings distributed throughout the whole Mediterranean Sea. Incidental capture by commercial fisheries seems to be the main threat for this species, followed by other anthropogenic lesions, such as head and carapace injuries, due to impacts with boats or propellers (Casale et al. 2003). There is little information about other causes of mortality in leatherback turtles and very few complete pathologic investigations have been carried out. Occasionally, *Photobacterium damselae* has been reported in stranded leatherback turtles from the Mediterranean (Poppi et al. 2012) as well as from other parts of the world (Obendorf et al. 1987). In this report, we present a pathological study of a stranded leatherback turtle from the western Mediterranean (Catalonian coast), with a severe necrotizing enteritis associated to *P. damselae*, showing that this bacterium may be a relevant pathogen for this species.

2. MATERIALS AND METHODS

On November 5th, 2016, an adult female leatherback turtle was found dead on the beach of Montroig (41º02’36’’N 1º00’07’’E), at the Catalonian Coast. The turtle was slightly autolyzed, with organs intact, and then given a D3 scoring for preservation (Flint et al. 2009). It was transported to the Veterinary School of the Autonomous University of Barcelona, where it was necropsied. Representative tissue samples were collected and preserved in 10% neutral buffered formalin for histopathological examination. After

48 hours of fixation, tissues were embedded in paraffin and routinely stained with hematoxylin and eosin. Additional stains (GRAM and Grocott) were performed to detect infectious agents in lesions. Microphotographs were taken with a Leica DM 6000 B Photomicroscope and a Leica camera DFC480, that added a scale bar automatically. Samples from intestinal mucosal were submitted for microbiological studies. Scrapped intestinal mucosa was inoculated onto Columbia agar with 5% sheep blood (Difco) and MacConkey agar (Oxoid) and incubated overnight at 37 °C in 5% CO_2. Investigation for Salmonella spp. was also performed following ISO guideline 6579-1:2017 (ISO 2017), with pre-enrichment culture of the sample in buffered peptone water (Oxoid) followed by selective enrichment in modified semi-solid Rappaport Vassiliadis medium (Oxoid). Salmonella-Shigella agar (Oxoid) and Xylose lysine deoxycholate agar (Oxoid) were used as solid selective media. Isolates were phenotypically identified using the API 20E and API20NE identification system (bioMérieux).

3. RESULTS

The turtle's curved carapace length was 126 cm and its curved carapace width was 91 cm, and its weight was 156 Kg. Upon external examination, a few small barnacles of the species *Chelonibia testudinaria* were detected (Epibiont Research Cooperative 2007). A large subcutaneous hemorrhagic and edematous area of about 15 x 10 cm was observed on the left flank of the animal, approximately 20 cm cranial to the hind limb. There were no other remarkable external traumatic lesions.

Approximately 3.5 liters of unclotted, dense, serosanguinous fluid were collected from the coelomic cavity, and two or three small filamentous blood clots were seen on the liver surface. There was a small amount of sand inside the larynx, trachea, and main

bronchi, apparently without causing occlusion (Fig.1A). The stomach contained a plastic refreshment straw and two short fragments (3-5 cm long) of thin fishing line, without apparent damage to the gastric mucosa. The serosal surface of small intestine was congested, and intestinal loops were turgid and slightly distended with gray-reddish dense fluid contents, up to the ileocecal sphincter (Fig. 1B). At this location there was a protruding firm diverticulum of about 3 cm in diameter. Distal to this ileocecal diverticulum, in the large intestine, there was almost no digesta, with few amounts of gas, indicating an obstructive effect at the level of the ileocecal diverticulum. The mucosa of the distal half of small intestine was uniformly thickened and there was a fine granular brown-orange material (identified as fibrin) diffusely attached to the mucosal surface, (Fig. 1C) effacing the normal honeycomb folds of the small intestinal mucosa of leatherback turtles (Fig. 1D). The ileocecal diverticulum was filled with a 3 cm diameter firm whitish dry mass, which was easily separated from the intestinal mucosa, that on cut surface was shown to contain concentric stratified material (Fig. 1E). No internal parasites were detected macroscopically.

Tissues were moderately autolyzed based on poor preservation of cellular morphologic details. Sections of small intestine revealed diffuse necrosis of the upper part of the mucosa (Fig. 2A), which was replaced by a thick band of hypereosinophilic cellular debris and fibrin (Fig. 2B). Large amounts of small rods were found adhered to and admixed with the necrotic cell debris (Fig. 2C). Most of these bacteria were Gram-negative, although some Gram-positive rods were also present. Moderate numbers of these Gram-positive rods were found in all organs investigated and were interpreted as overgrowth of saprophytic bacteria due to autolysis. The mass found in the ileocecal diverticulum corresponded to a granulomatous-necrotizing exudate, disposed in

concentric layers with bands of hypereosinophilic necrotic cells (consistent with degenerated macrophages and heterophils) along with fibrin, necrotic material, and bacteria (Fig. 2D). No significant lesions were observed in the other organs investigated (liver, lung, spleen, brain). No fungal structures were detected with Grocott stain.

After 24h, a pure culture of β-hemolytic Gram-negative rods was obtained from cultured scrapped mucosa of affected small intestine. The isolated bacterium was identified as *P. damselae* with a percentage of identity of 99.9% using the API 20E (profile 6015004) and API20NE (profile 5300044) identification system. *P. damselae* includes two subspecies: *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida,* which exhibit discriminating biochemical/phenotypical features. According to the positive results obtained for β-hemolysis, nitrate reduction, urease, and lysine decarboxylase, our isolate was identified as *P. damselae* subsp. *damselae.* No *Salmonella* spp. were recovered.

4. DISCUSSION

Infections with bacteria from the family *Vibrionaceae* are frequent in sea turtles, with most of these infections being caused by bacteria of the genus *Vibrio* (Glazebrook et al. 1981, Work et al. 2003, Orós et al. 2005, Zavala-Norzagaray et al. 2015). However, bacteria from the genus *Photobacterium*, another of the eight genera included in the family, are not so frequently isolated from sea turtles. The taxonomy of the genus *Photobacterium* has been recently reviewed (Labella et al. 2017). It contains 27 species with valid names, with *P. damselae* being the most pathogenic for marine vertebrates. Two subspecies of *P. damselae* are described, and both have been implicated in enteritis in leatherback turtles. *Vibrio damsela* (*P. damselae* subsp. *damselae* under the actual taxonomy) caused diphteric enteritis, valvular thrombotic endocarditis, and septicemia

in a female leatherback turtle stranded in Tasmania, Australia (Obendorf et al. 1987). *P. damselae* subsp. *piscicida* (Gauthier et al. 1995) was determined to be the cause of death of a stranded leatherback turtle with acute hemorrhagic gastroenteritis in the Adriatic Sea (Poppi et al. 2012). Recently, P. *damselae* subsp. *damselae* has been isolated from intestine and other organs from three stranded loggerhead turtles (*Caretta caretta*) in the Tuscany coast of Italy (Fichi et al. 2016). Virulence of *P. damselae* subsp. *damselae* is based on the secretion of toxins like phospholipase-D Dly (damselysin) and the pore-forming toxins HlyApl and HlyAch (Rivas et al. 2013). The stranded leatherback turtle presented in this report had a diffuse fibrino-necrotizing enteritis and obstructive ileocecal diverticulitis, and *P. damselae* subsp. *damselae* was isolated in pure culture from a mucosal scrapping, corroborating the pathogenicity of this bacteria for leatherback turtles. The relationship between the observed fibrino-necrotizing enteritis and the ileocecal diverticulitis is uncertain, but probably both lesions are unrelated to each other. The existence of a diverticulum at the ileocecal junction appears to be an anatomical feature of leatherback turtles, not present in other sea turtle species (Magalhães et al. 2012). The diverticulum appears to be well developed in adult individuals, but not in juveniles, and a high number of turtles showed inflammatory changes of the diverticulum, which become distended by exudate. However, in none of their cases the diverticulitis appeared to be associated to other lesions in small or large intestine (Stacy et al. 2015). In our case there was intestinal obstruction at the ileocecal junction, a complication not recorded previously in association with diverticulitis. The intestinal lumen becomes narrowed in cases of diverticulitis (Stacy et al. 2015), therefore we suggest that the thickening of the intestinal mucosa due to the fibrino-necrotizing exudation in concurrence with diverticulitis have led both together to

obstruction. This view is supported by the fact that another leatherback turtle necropsied in our laboratory in 2016 (data not shown) had also diverticulitis in absence of other enteric lesions (Fig.1F).

P. damselae subsp. *damselae* is a water transmitted (Fouz et al. 2000) pathogen of marine fishes, including blacksmith (*Chromis punctipinnis*), sharks, turbot (*Scophthalmus maximus*), yellowtail (*Seriola quinqueradiata*), red-banded sea bream (*Pagrus auriga*), sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), in which it causes granulomatous ulcerative dermatitis (Austin 2010, Khouadja et al. 2014). It has been also isolated from healthy skin and wounds from cetaceans, including bottlenose dolphin (*Tursiops truncatus*) and Bryde's whale (*Balaenoptera brydei*). Its role as a human pathogen is limited to causation of wound infections (Rivas et al. 2013).

In addition to the enteric bacterial disease, there was a bruise on the turtle's left side, with subcutaneous hematoma, caused most likely by a blunt trauma to this side of the body, possibly by a vessel strike. The excessive accumulation of serosanguinous fluid in the coelomic cavity was probably a consequence of intestinal serositis. Hepatic rupture may have increased the amount of serosanguinous fluid, although liver rupture and hemorrhage were not clearly seen, and the liver was autolyzed and friable upon removal, making it very difficult to distinguish lesions from postmortem alterations. Parasitism is a frequent cause of morbidity and mortality in sea turtles (Greiner 2013) but no significant parasitic diseases were observed in this case.

Sand is thought to have been introduced into the airways peri- or post-mortem as the animal was tossed onto the beach by wave action. Despite many studies establishing the ingestion of marine debris as a major threat to marine turtles (Bugoni

et al. 2001, Mrosovsky et al. 2009), in this case few foreign bodies were present in the gastrointestinal tract, and those found were not thought to be associated with disease.

In view of the severity of intestinal lesions observed in our case, as well as of previous reports of disease by others, the capability of *P. damselae* subsp. *damselae* to act as a primary agent of disease in stranded or sick leatherback turtles should be considered.

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FIGURES

Fig. 1. Gross findings of stranded leatherback turtle necropsy. (A) Deposits of sand in tracheal lumen (B) Distention of small intestine and reddening of the intestinal serosa (esophagus and stomach on the left side). Arrowhead is showing the transition of small intestine to large intestine. Large intestine was distended with gas. (C) Mucosal surface of small intestine showing fibrino-necrotizing enteritis. The surface has been rinsed with water (D) Normal small intestine mucosa of another leatherback turtle showing the characteristic honeycomb pattern (E) Section of the luminal nodulation found in the ileocecal diverticulum, composed of white stratified material. (F) Normal leatherback turtle showing transition of small intestine (left) to large intestine (right), and ileocecal diverticulum (arrowhead) with diverticulitis (presence of a spherical stratified mass composed of fibrin and cellular exudate, see Stacy et al., 2014).

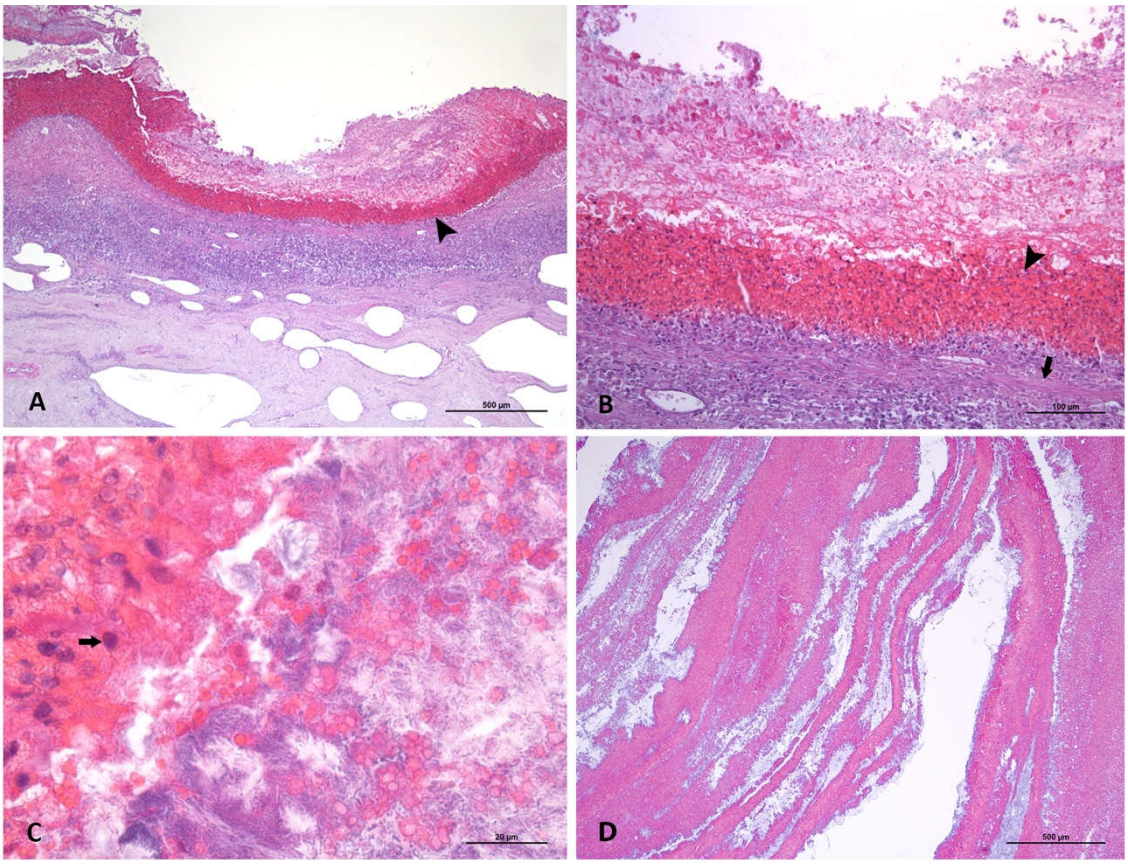

Fig. 2. Small intestine, leatherback turtle, Haematoxylin & Eosin staining. (A) Intestinal mucosa and submucosa, low magnification, showing a fibrino-necrotizing enteritis, with a superficial layer (arrowhead) of necrotic tissue and fibrin. Necrosis extends through almost whole mucosa. Submucosal vessels are distended by gas from autolysis (B) Small intestine, mucosa, closer view of fibrino-necrotizing enteritis (arrowhead). There is a layer of bright eosinophilic necrotic tissue underneath a loose layer of fibrin and cell debris mixed with clumps of basophilic rods. Basophilic, pyknotic cell nuclei are still visible in the necrotic layer. The muscularis mucosae is visible underneath the necrotic mucosa (arrow). (C) Detail of fibrino-necrotic mucosa (upper-left side of the photograph), with pyknotic cell nuclei, and numerous bacteria overlying the necrotic enteric mucosa. Most of these bacteria were GRAM-negative, but also some large GRAM-positive rods were present. (D) Diverticular mass showing stratified layers of inflammatory infiltrates, cellular debris and intestinal contents with bacteria.