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INVESTIGATIONS ON THE PATHOGENICITY OF NON-CHOLERIC *VIBRIO* SPECIES, ISOLATED IN AQUATIC TURTLE

INVESTIGAȚII ASUPRA PATOGENITĂȚII SPECIILOR *VIBRIO* NON-HOLERICE, IZOLATE LA BROAȘTELE ȚESTOASE ACVATICE

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ABSTRACT | REZUMAT

We have undertaken comparative research between species of aquatic turtles from the subtropical areas of America from the genera *Trachemys* and *Chrysemys* and the species from the European temperate zone, *Emys orbicularis*. These studies consisted of the sampling of digestive and respiratory flora from which bacterial strains belonging to the *genus Vibrio* were isolated, with a particular focus on the isolation of *non-choleric vibrios*. Research was carried out on the comparative analysis of the clinical, necropsy, and histopathological results that followed the research. Research has shown that the most pathogenic strains are *V. vulnificus* and *V. parahaemolyticus*. Regardless of the pathogenetic pathway, they cause damage to the liver, pancreas, and intestines, sometimes with serious histopathological changes, together causing death. In the same studies, it was proven that the presence of non-choleric *Vibrio spp*. species in aquatic turtles implies the possibility of animals in aquatic ecosystems to develop morbid entities, themselves evolving in the storage of vibrios and ensuring the enrichment of water with these bacteria. This state of carrier and eliminator keeps vibrios in the circuit of aquatic ecosystems for a long time, even in periods of too low or too high temperatures. These conditions that can destroy the vibrios in the water are overcome as a possibility for their survival by the fact that the vibrios find in the aquatic creatures a true biological niche that ensures their permanence and spread in the ecosystem.

Keywords: Vibrio's, bacterial stems, aquatic tortues, pathogenity, lesions

Am întreprins cercetări comparativ între speciile de țestoase acvatice din zonele subtropicale ale Americii din genurile *Trachemys* și *Chrysemys* și specia din zona temperată europeană *Emys orbicularis*. Aceste studii au constat în prelevarea de floră digestivă și respiratorie din care s-a făcut izolarea de tulpini bacteriene încadrate în genul *Vibrio*, urmărindu-se în special izolarea de vibrioni non-holerici. Au fost realizate cercetări privind analiza comparativă a rezultatelor clinice, necropsice și histopatologice care au urmat cercetărilor. Cercetările efectuate au arătat că cele mai patogene tulpini sunt *V. vulnificus* și *V. parahaemolyticus*. Acestea determină, indiferent de calea patogenetică, leziuni ale ficatului, pancreasului și intestinelor, uneori cu modificări histopatologice grave, provocând împreună decesul. În aceleași studii s-a dovedit că prezența speciilor de *Vibrio* spp. non-holerice la țestoasele acvatice implică posibilitatea animalelor din ecosistemele acvatice de a dezvolta entități morbide, ele însele evoluând în stocarea de vibrioni și asigurând îmbogățirea apei cu aceste bacterii. Această stare de purtător și eliminator menține timp îndelungat vibrionii în circuitul ecosistemelor acvatice, chiar în perioadele de temperatură prea scăzută sau prea ridicată. Aceste condiții care pot distruge vibrionii din apă sunt depășite ca posibilitate de supraviețuire a acestora, prin faptul că vibrionii găsesc în creaturile acvatice o adevărată nișă biologică care le asigură permanentizarea și răspândirea în ecosistem.

Cuvinte cheie: *Vibrio*, tulpini bacteriene, broaște țestoase acvatice, patogenitate, leziuni

The topicality and general interest raised by vibriosis is demonstrated by the fact that these diseases are more and more frequently in the attention of bacteriologists, epidemiologists, and clinicians. On the subject of infections produced by these bacteria, especially non-choleric *Vibrio* species, some studies have been published in recent years, and research in this field has gained a special scope (8-12).

In the last decade of the last millennium, many researchers drew attention to the fact that non-choleric *Vibrio* species are frequently involved in food poisoning in humans, manifested by acute diarrheal syndrome. Although, from a clinical point of view, these vibriosis do not present complications and are effectively treated, the epidemiological risk is important, with episodes involving hundreds of cases (1, 2, 4, 6).

The increased prevalence of the isolation of non-choleric *Vibrio* species from various over-infected external wounds naturally led to the statistical study of the frequency of isolation of these bacteria from water,

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sediments, and different aquatic animals. Thus, Oliver et al. (1995) demonstrated that there is a direct link between *Vibrio* species and aquatic organisms located on different levels of the trophic pyramid (7, 8, 11). We considered it necessary to study how vibrios contaminate aquatic animals, possible clinical manifestations, and the role of vibrios-contaminated aquatic animals in the enrichment of water with bacteria. Initially, we used only native aquatic turtles of the species *Emys orbicularis* for the studies. This species, although a frequent carrier of non-choleric vibrios, proved a natural increased resistance to the infection, with clinical signs being rarely observed and the anatomopathological changes being reduced. Rare cases clinically expressed short periods of inappetence, in all cases presenting the state of carrier and eliminator of vibrios for a maximum of 7 days (9, 10). Later, the studies were also developed on species of aquatic turtles belonging to the genera *Trachemys* and *Chrysemys*, which are frequently found as pets (10).

MATERIALS AND METHODS

The studies included species of aquatic turtles with the following taxonomic classifications: Order *Vertebrata*, Suborder *Gnathostomata*, Superclass *Tetrapoda*, Class *Reptilia*,Subclass *Anapsida*, Superorder *Chelonia*, Order *Cryptodira*, Family *Emydidae*, Subfamily *Emydinae*, Genus *Chrysemys*, Genus *Trachemys*, and Genus *Emys*, *Emys orbicularis* species.

The samples were processed by special methods for isolating and identifying the bacterial species included in the *Vibrio* genus. The methods generally followed the stages stipulated by standardised methods (STAS ISO 8914, FAO-OMS methods) in order to identify *Vibrio parahemolyticus* and other *Vibrio* species in food and biological samples. The method was adjusted to reveal all the *non-choleric vibrio's* that might exist in the samples. Many samples were processed using the method suggested by James (1995) and Tudor et al. (2001) (3, 5, 9, 10). The confirmation was made by the PCR method (9, 10). The samples were prepared, and the content was extracted and homogenised, afterwards being obtained in 1:1 and 1:10 dilutions in alkaline buffered water (ABW) through their introduction in 50 and 180 mL of ABW, respectively. The samples (0.2 g from the 1:1 dilution and 0.1 mL from the decimal dilution) were distributed in plates with agar containing 1% tryptone and 3% sodium chloride, in duplicate. These plates were further incubated at 37°C for 18–24 hours.

The *DNA extraction:* the bacterial cultures and the crustacean and shellfish samples were centrifuged at 5000 rpm for 10 min, and the sediment was collected using a QIAamp DNA kit. The DNA quantity and its purity were determined using spectrophotometry, obtaining the percentages for 260/280 nm. The concentrated DNA (a result of the ethanol precipitation) was subjected to vacuum evaporation through centrifugation. The sediment was suspended again in 50 or 100 µl of Tris-EDTA buffer solution (pH = 8; 10 mM Tris, 1 mM EDTA) and incubated at 65°C for 10 min in order to solubilize the DNA. The concentrated DNA was stored at -20°C (9, 10).

REZULTS AND DISCUSSIONS

All individuals included in the study were followed to detect pre- and post-therapeutic clinical signs. At the same time, the studies aimed to demonstrate the possibility that some species of aquatic animals. Although they do not develop clinical symptoms, they become carriers and eliminators of vibrios, sometimes for long periods. In this sense, post-therapy samples were taken until the moment of negating the presence of vibrios. The clinical signs on the basis of which the infection was suspected were: soft faeces with a tendency to dissociate (diarrhoea), inappetence, apathy, the appearance of haemorrhagic changes in the skin, carapace or plastron. Haemorrhagic changes were not found in *Emys orbicularis*; instead, in this species, a dissociation of the joining plates at the level of the plastron was noted, associated in many situations with a decrease in its specific resistance or in density.

Digestive samples (especially cloacal, deep cloacal, and oesophageal) and samples from the upper respiratory system were collected. In parallel, samples were collected from the ambient water to determine the possibility of vibrios recovery and their persistence in the environment. The samples were collected at a frequency of 7 days until the negative presence of vibrios in the ambient water samples. In this way, the possibility that following the application of the therapeutic behaviour and the remission of the symptoms, if recontamination of the treated individuals may occur. Following the application of therapeutic treatment to 27 *Emys orbicularis* individuals found positive for non-choleric vibrios infection, 22 clinical subjects were recovered, with the other 5 subjects reaching exitus in different phases of the disease's evolution. Early application of therapy led to faster recovery for individuals. The clinical subjects in whom the therapeutic intervention was done in the advanced phases of the disease evolution needed prolonged treatments, the recovery being difficult, and the individuals presenting sequelae for long periods of time (months) both at the respiratory and digestive levels.

The therapeutic intervention in American aquatic turtle species was carried out in 54 positively diagnosed individuals, of whom 42 were recovered, with the other 12 subjects reaching exitus in different phases of the disease's evolution. It should be noted that

the evolution of the disease was much more severe in all clinical cases, compared to European clinical subjects. From an anatomopathological point of view, following the necropsy of fatal cases, lesions were found at the level of the digestive tube, and very rarely lesions at the renal or splenic level. The main morphopathological dominants were gastroenteritis (serous, haemorrhagic, haemorrhagic-necrotic, or necrotic) and, more rarely, colitis (usually haemorrhagic-necrotic colitis). In many cases, liver congestion, or miliary liver abscesses, was found. Following the necropsy of cases with chronic evolution, although the clinical symptoms had been completely remitted, serous gastroenteritis (sometimes even haemorrhagic) and hepatitis were still found.

Fig. 1. Chronic haemorrhagic state at the level of the plastron, highlighting the junctions

Fig. 2. Haemorrhagic condition at the level of the carapace and the obvious damage to the scales

In turtles from the subtropical zone, after necropsy, hepatopancreatitis and degeneration of the hepatopancreas were frequently found. Activation of Kupffer cells, mononuclear infiltrate, and hemosiderin deposits were found in the liver, and only in subtropical aquatic turtles in most cases degenerative hepatopancreatitis was found. Gastric lesions were dominated by haemorrhagic gastritis and oedema of the submucosal layers, sometimes haemorrhagic-necrotic gastritis. In the small intestine, the dominant lesions were haemorrhagic-necrotic enteritis, necrosis and detachment of the mucosa from the underlying layers, mononuclear infiltrate in the chorion, and vacuolization of neurons in the myenteric plexus. In the large intestine, hyperplasia of goblet cells and detachment of the mucosa, mononuclear infiltrate with a tendency to organization, desquamation of enterocytes were noted. In a few cases, catarrhal enteritis with tearing of the muscle layer through transudation phenomena, necrotic enteritis with desquamation of the villous epithelium and numerous mononuclear infiltrations in the chorion, haemorrhages in the intestinal mucosa and vacuolar-type degeneration at the liver level, hepatocytic reactions with granular-type degeneration, and with the appearance of vesicular nuclei were found.

Fig. 3. Generalised haemorrhagic lesions in tissues and organs

CONCLUSIONS

The evolution of infections with non-choleric *Vibrio* species in aquatic turtles determined the rapid and intense increase in the number of heterophiles and a slight increase in the number of monocytes. The non-choleric vibrio species isolated were *V. parahaemolyticus*, *V. alginolyticus,* and *V. vulnificus*. All isolated species determined immune reactions of medium intensity highlighted by blood biochemistry analyses.

The clinical evolution was much worse in aquatic turtles from the sub-tropical zone compared to the species from the temperate zone. The aggravating symptomatology with a tendency to become chronic is the reason for the higher rate of therapeutic success in Emys orbicularis (81.18% saved and 18.52% exitus) compared to subtropical aquatic turtles (77.78% saved and 22.22% exitus). The anatomopathological changes were more drastic in aquatic turtles from the sub-tropical zone compared to the species from the temperate zone. Due to the serious damage to the hepatopancreas and kidneys, even if the symptoms were in remission, after an interval of a few weeks, the exitus was reached.

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