FATAL BACTERIAL INFECTION OF THE HEART INCAPTIVE BOTTLENOSE DOLPHIN (TURSIOPS TRUNCATUS GILLI). A CASE STUDY.

T. Tserodze1,2, D. Jgenti1, M. Mgeladze1, N. Chkheidze1, R. Lomidze1, J. Gambashidze1, N. Janelidze3, E. Didebulidze3, A. Groene4, M. Kik4 and M. Tediashvili3.
1. Black Sea Flora and Fauna Research Center, Batumi, Georgia.
2. Rustaveli Batumi State University, Batumi, Georgia.
3. G. Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia.
4. Faculty of Veterinary Medicine, Utrecht University, Netherlands.

Introduction:
In recent years the Pacific Bottlenose dolphins (Tursiops truncatus gillii) are often found in noogenic environments. Modern dolphinariumsand/or aquariums are now considered as much more relevant and safe living environment for their habitats. Despite of continuous monitoring done at the dolphinariums for animal health parameters, also water and food qualitythe captive marine mammals are still vulnerable to changes in the enviroment orI infectious agents that can lead even to a fatal outcome. There have been many individual cases reported on dolphin’s illness and death cases which can be due to different reasons, including diseases of infectious etiology (Song et al, 2017) Bacterial cultures obtained from marine mammals often yield in multiple genera and species, and it is ususally difficult to determine if a cultured bacterium is a primary pathogen (Venn-Watson et al at al, 2008).

Key words:- Marine mammals, dolphinarium, necropsy, hystopathological findings, heart, bacterial infection, Staphylococcus aureus, E. coli
A number of detailed studies have been done to determine the prevalent illness types and the relative risk of various bacteria for marine mammals, including Atlantic bottlenose dolphins. Bacterial infections such as septicemia, pneumonia, meningitis, myocarditis, peritonitis, enteritis have been found to be prevalent causes of death for adult dolphins. McFee et al (2009) analyzed the major pathologic findings and probable causes of death of bottlenose dolphins over a 14-yr period (1993–2006) in the coastal region of South Carolina and found out that of about 31% of animal likely died of infectious disease and most of these were septicemias and/or bacterial pneumonias. The diagnoses were based on histopathology, bacterial cultures were not routinely performed. In the frames of US Navy Marine Mammal Program (Venn-Watson et al, 2008) 20 years retrospective data on microbially isolate cultured from dolphins internal organs or fluid samples were analyzed. Highest risk bacterial isolates were most likely to be identified in pleural fluid, followed by renal and splenic tissues. Staphylococcus aureus was identified as a highest risk bacterial pathogen in the studied dolphin population, accounting for 0.4% of total bacterial isolates. At the same time, no sole bacterial isolate was definitively associated with morbidity and mortality in marine mammals. Other studies conducted in different years on Bottlenose dolphins (Streitfeld MM, Chapman CG, 1976; Venn-Watson et al, 2008) also indicated presence of several bacteria, with prevalence of S. aureus as a high risk pathogen. The investigation carried out on the capture-release dolphins at multiple sites of the US, such as Mid-Atlantic coast and Gulf of Mexico, and also estuarine waters of south-Eastern coast (Schaefer et al, 2009) revealed Vibrio, Escherichia coli, Plesiomonas shigelloides, Aeromonas hydrophila, Shewanella putrefaciens, Pseudomonas fluorescens/putida, also S. aureus as the most common bacteria. In one of the earlier reports (Liong et al, 1985) a multisystemic inflammation of Tursiops truncatus including suppurative enteritis, encephalitis, and pneumonia with chronic pancreatitis was described related to Meliodosis (Bukholderia pseudomallei) as a documented infection which lead to a rapid progressive respiratory distress and fatal septicemia in spite of antibiotic therapy. Bacterial pneumonia has been also found to be common in other studies of dolphin mortality (Howard et al., 1983). Brucella-induced placentitis has been reported in bottlenose dolphins (Miller et al., 1999). Recently, Beta-hemolytic gram positive cocci (Streptococcus iniae - fish pathogen), were isolated first time from the captive bottlenose dolphin in China (Song et al, 2017). Mortality in the first 3 months after birth is a serious problem in captive bottlenose dolphins. In one of the earlier reports (Tserodze et al, 2016), the regular monitoring of animals health status has been performed based on daily observation by qualified marine mammals trainers and veterinarians, along with routine blood tests (complete blood analyses and also blood biochemical parameters), also cytological and bacteriological analysis of dophin’s exhaled air and gastric juice. The high quality balanced food, rich in vitamins and minerals ensured adequate health status preservation of the marine mammals in this particular noogenic environment. Additional determination of the above mentioned parameters has been performed according to the needs, in particular, if any change in animal’s behavioral traits such as passive swimming, rapid breathing, irritating factors and inadequate reaction of the others was identified.

Here we describe a case of the fatal illness of adult bottlenose dolphin (T. truncatus gillii) nicknamed “Kako”, inhabitant of the Batumi Dolphinarium in Georgia.

Case report:-

At the beginning of 2011, seven Pacific Ocean dolphins (two males and 5 females) including the object of our report, dolphinine "Kako", were purchased in Japan (Island Taiji) and brought to the Dolphinarium in Batumi, Georgia. After the primary adaptation period the animals were kept in a controlled living environment, where chemical and bacteriological parameters of water quality were routinely checked and adjusted in case of need according to the guidelines (Tserodze et al, 2016). The regular monitoring of animals health status has been performed based on daily observation by qualified marine mammals trainers and veterinarians, along with routine blood tests (complete blood analyses and also blood biochemical parameters), also cytological and bacteriological analysis of dolphin’s exhaled air and gastric juice. The high quality balanced food, rich in vitamins and minerals ensured adequate health status preservation of the marine mammals in this particular noogenic environment. Additional determination of the above mentioned parameters has been performed according to the needs, in particular, if any change in animal’s behavioral traits such as passive swimming, rapid breathing, irritating factors and inadequate reaction of the others was identified.

After the primary adaptation period the animals were kept in a controlled living environment, where chemical and bacteriological parameters of water quality were routinely checked and adjusted in case of need according to the guidelines. The regular monitoring of animals health status has been performed based on daily observation by qualified marine mammals trainers and veterinarians, along with routine blood tests (complete blood analyses and also blood biochemical parameters), also cytological and bacteriological analysis of dolphin’s exhaled air and gastric juice. The high quality balanced food, rich in vitamins and minerals ensured adequate health status preservation of the marine mammals in this particular noogenic environment.

From beginning of 2011 to February 2017, "Kako" dolphin’s health condition was satisfactory (Tables 1 and 2), except for one episode that took place on January 31, 2015, when the general blood analysis of the animal showed leukocytosis and some changes in blood biochemical indicators without any notable clinical symptom. However, appropriate antibiotic therapy was initiated and after two weeks blood parameters returned to normal values.

On February 18, 2017, the routine monitoring in Batumi dolphinarium, revealed negative changes in the exhaled air of Dolphin “Kako”, in particular, in exhaled air sample high number (>1000 CFU/ml) of nonhemolytic bacterial colonies were registered. Gram stain and microscopic examination of selected prevalent colonies showed gram-positive cocci, predominantly staphylococci, also gram-negative rod-shaped bacteria. The general blood analysis of the “Kako” dolphin conducted on 23rd February, 2017 demonstrated leukocytosis (15.740 k/ml) with increased
neutrophil counts (76.5%). However all other blood parameters were normal, as well as most of the blood biochemical indicators, except bilirubin (Table 3.4). The analysis of the gastric juice also didn't reveal any abnormality. The behavioral characteristics of the “Kako” dolphin were quite satisfactory. Thus no medicinal intervention was carried out and the observation has been continued according to the standard scheme.

**Table 1:** The blood clinical parameters of dolphin “Kako” in 2011-2016.

| Common Blood Test | Units  | Results of blood analysis from 2011 to 2016 (minimum-maximum) | Data contributed by SeaWorld Clinical Laboratory (Bossart, 2001) |
|-------------------|--------|---------------------------------------------------------------|---------------------------------------------------------------|
|                   |        | Free ranging Bottlenose Dolphins                              | Free ranging Bottlenose Dolphins                              |
| RBC               | m/mkl  | 3.35-4.36                                                    | 3.1-4.0                                                       |
| HGB               | g/dl   | 14.3-17.4                                                    | 12.7-15.5                                                     |
| HCT               | %      | 43.36-51.94                                                 | 37-47                                                        |
| MCV               | fl     | 116-136                                                      | 111-127                                                      |
| MCH               | pg     | 38-47.5                                                      | 36-46                                                        |
| MCHC              | g/dl   | 32.5-36.4                                                   | 32-35                                                        |
| PLT               | k/mkl  | 68-162                                                      | 92-217                                                       |
| RET%              | %      | 0-1.2                                                       | -                                                            |
| ESR               | mm/sT  | 2-4                                                        | 1-2.3                                                        |
| WBC               | k/mkl  | 6-12.02                                                        | 5.6-12.4                                                      |
| BAND NEU          | %      | 0-2                                                       | 0                                                            |
| NEU               | %      | 30-65                                                        | 45.4-49.5                                                     |
| LYMPH             | %      | 12-32                                                        | 9.3-19.5                                                     |
| MONO              | %      | 0.5-4                                                       | 1.4-4.9                                                      |
| EOS               | %      | 14-43                                                        | 13.2-36.5                                                    |
| Baso              | %      | 0                                                           | 0                                                            |

**Table 2:** The blood average biochemical parameters of dolphin "Kako" in 2011-2016.

| Common Blood Test | Units | Data of blood analysis from 2011 to 2016 (minimum-maximum) | Data contributed by SeaWorld Clinical Laboratory (Bossart, 2001) |
|-------------------|-------|---------------------------------------------------------------|---------------------------------------------------------------|
|                   |       | Free ranging Bottlenose Dolphins                              | Captive Bottlenose Dolphins                                  |
| ALT               | U/l   | 21-56                                                       | 9.0-33                                                       |
| AST               | U/l   | 177-426                                                      | 133-318                                                      |
| GGT               | U/l   | 37-78                                                       | 17-31                                                        |
| ALP               | U/l   | 129-511                                                      | 51-610                                                      |
| CK                | U/L   | 85-257                                                      | -                                                            |
| TP                | g/dl  | 6.9-8                                                        | 6.4-8.8                                                      |
| ALB                | g/dl  | 4.2-5.1                                                     | 2.9-3.7                                                      |
| GLOB              | mg/dl | 2.2-3.3                                                     | 3.1-5.5                                                      |
| BIL – T           | mg/dl | 0.3-1                                                       | 0.1-0.4                                                      |
| Glucose           | mg/dl | 58-92                                                       | 62-139                                                      |
| CREA              | Umol/L| 1-1.9                                                       | 1-2.1                                                        |
| BA                | mg/dl | 5-30                                                        | -                                                            |
| CHOL              | mg/dl | 146-184                                                      | 137-235                                                      |
| BUN               | mmol/L| 44-58                                                       | 45-72                                                        |
| K                 | mg/dl | 3.7-4.9                                                     | 3.2-4.4                                                      |
| Ca -T             | mmol/L| 9.6-11.1                                                    | 8.2-9.4                                                      |
| Na                | mg/dl | 143-154                                                     | 151-158                                                      |
| Mg                | U/l   | 1.7-2.3                                                     | -                                                            |
| Phos              | U/l   | 4.3-6.4                                                     | 3.2-7.2                                                      |

In the first week of March 2017 the next series of analyses done for “Kako” dolphin demonstrated the similar condition, with slight positive changes (table 3.4). However, antibacterial and immunomodulating treatment was...
initiated to control the potential infectious process. More specifically, a 10 days treatment course with the antibiotic amoxicillin clavunate (“Clavomed” - World Medicine Limited; Great Britain) 1000 mg per day (2 x 5mg / kg) for 10 days, and the immune modulatory preparation “Broncho-munal® P 7mg” (Om Pharma, Switzerland, Geneva) 2 capsules / day for 10 days was conducted. The monitoring scheme for "Kako" dolphin’s health status was also slightly modified: the bacteriological inspection of the animal’s exhaled air has been done once in two weeks and the blood test- once a month. Despite the fact that the behavioral characteristics of the animal were evaluated as normal, the some blood indicators still indicated the possibility of bacterial infection. On 05 April 2017 the following changes were introduced in the treatment scheme: a 5-day course of erithromycin (PolfaTarchomin S.A., Poland) (1.0 g 2 times a day) started in parallel with the EchinaceaCompositeum(BiolegisheHeimmittl Heel, Germany) (1 ampula 1 x day, 14 days). In the same period next round of 10-day course of Bronchomunal was conducted along with the liquid levokarinit10 ml (MagisFarmaceutici,Brescia) (prescribed as 1 ampule per day for 3 days).

The next round of dolphin “Kako”’s blood tests conducted on 28.04.2017 showed persistent leukocytosis (13.97 /mkl) with increased neutrophil counts (71%), and still normal biochemical parameters, with only increased bilirubin value (1.1 mg/dl). Since complex medicinal therapy conducted so far did not produce positive results, the new treatment has been started, in particular the ciprofloxacin (Ultra laboratories PVT.LTD Bangalore, India) 5 g per day (15 mg / kg) for 10 days, and hepatoprotector hepatrine(Evalar, Russia) (2 capsules per day) for 2 months. Bacteriological analyses done during the treatment period (on 04.05.2017 and 18.05.2017) showed again extensive bacterial growth (>1000 CFU hem and >10000 CFU hem; accordingly) in “Kako”’s exhaled air sample. The 3-rd round of treatment with immunomodulator “Bronchomunal” was conducted.

Samplings done on 23.05.2017 showed bacterial overgrowth and leukocytosis, which indicated continuous respiratory infection along with signs of possible hepatitis. At that stage, in addition to the carried treatment a one month course (2 ampoules per day) of the metabolic regulator “Myocardin (levokarinit)” was given together with the 10-day repeated course of ciprofloxacin. It is noteworthy that again no negative change in animal’s behavior has been observed.

### Table 3: The blood clinical parameters of dolphin "Kako" (selection from the period of 23.02.2017 to 22.07.2017)

| Common Blood Test | Units | Normal range | 23.02.2017 | 06/03.2017 | 06/04.2017 | 28.04.2017 | 20.06.2017 | 02.07.2017 | 22.07.2017 |
|-------------------|-------|--------------|------------|------------|------------|------------|------------|------------|------------|
| RBC               | m/mkl | 3.0 - 3.7    | 4.23       | 4.24       | 4.36       | 4.14       | 4.01       | 3.95       | 3.72       |
| HGB               | g/dl  | 13.5 - 15.5  | 17.3       | 15.5       | 16.1       | 15.3       | 14.9       | 14.1       | 12.3       |
| HCT               | %     | 38 - 44      | 48,56      | 49,06      | 49,81      | 47,55      | 46         | 45         | 38,92      |
| MCV               | fl    | 115 - 135    | 115        | 116        | 114        | 115        | 115        | 112        | 105        |
| MCH               | pg    | 38 - 48      | 40,8       | 36,6       | 36,9       | 36,9       | 37,1       | 36,2       | 33,1       |
| MCHC              | g/dl  | 34 - 36      | 35,5       | 31,7       | 32,3       | 32,2       | 32,4       | 31,2       | 31,6       |
| PLT               | k/mkl | 80 - 150     | 95         | 68         | 111        | 118        | 111        | 105        | 205        |
| ESR               | mm/sT | 4,0 - 17,0   | 2          | 4          | 4          | 2          | 3          | 35         | 60         |
| WBC               | k/mkl | 5,0 - 9,0    | 15,74      | 13,77      | 12,68      | 13,97      | 15,44      | 15,9       | 17,19      |
| Banded NEU        | %     | 0            | 0          | 1          | 0          | 1          | 0          | 1          | 0          |
| NEU               | %     | 53.8 - 64.6  | 76         | 68         | 58         | 71         | 68         | 72         | 56         |
| LYMPH             | %     | 16.8 - 18.4  | 9          | 7          | 10         | 7          | 15         | 14         | 19         |
| MONO              | %     | 2.8 - 3.8    | 1          | 1          | 1          | 1          | 1          | 1          | 1          |
| EOS               | %     | 10.6 - 11.3  | 14         | 23         | 31         | 20         | 16         | 13         | 24         |
| Baso              | %     | 0            | 0          | 0          | 0          | 0          | 0          | 0          | 0          |

### Table 4: The blood biochemical parameters of dolphin "Kako" (selection from the period of 23.02.2017 to 22.07.2017)

| Blood Biochemistry | Units | Normal range | 23.02.2017 | 06/03.2017 | 06/04.2017 | 28.04.2017 | 20.06.2017 | 02.07.2017 | 22.07.2017 |
|--------------------|-------|--------------|------------|------------|------------|------------|------------|------------|------------|
| ALT                | U/l   | 28 - 60      | 34         | 38         | 29         | 48         | 37         | 37         | 28 - 18    |
| AST                | U/l   | 190 - 300    | 289        | 310        | 299        | 358        | 279        | 265        | 265        |
| GGT                | U/l   | 30 - 50      | 49         | 51         | 53         | 51         | 52         | 49         | 48         |
The next blood analysis done on June 2, 2017, showed a tendency of increase in WBC number (15.44 k/mkl) with high content of neutrophils (68%); The blood biochemical indicators were within normal range but increased value of bilirubin (1.2 mg/dl). During the same period the exhaled air bacteriology (04.06.2017) revealed high bacterial growth (>10000). The animal’s behavior wasn’t changed substantially. Correction in the treatment scheme was done based on the antibiogram of the total mixed bacterial culture and a 14-day course of erythromycin (1.0 g x2 day, 5 mg / kg) was prescribed.

Next round of laboratory investigations (18.06.2017) of “Kako” dolphin showed that the treatment did not yield positive results despite the conducted antibiotic therapy, although the behavior of the animal did not change substantially. In the Dolphin’s exhaled air high bacterial growth (10^5 CFU) was registered. On the basis of the antibiogram of the mixed culture the treatment scheme has been modified once again and one-month course of Rifampicin (“BarshagovskiiPharameceutical Plant”, Ukraine) was prescribed (0.5 g x2 day, 2.5 mg/kg PO BID). The hematological investigation of “Kako” dolphin done on 02.07.17 demonstrated leukocytosis (15.9 k/mkl) with neutrophilosis (72%) as well as the increase in erythrocyte sedimentation rate (ESR) (35 mm / h) – again without significant changes in animal behavioral. The antibiotic therapy (rifampicin) in this period was continued and in addition Hepatrin (Evalar, Russia) and Avilac (Amvilab, USA, Atlanta) were given to the animal for the prevention of gastrointestinal system disorders. During 11.07.17 - 21.07.17 a minor change was observed in the animal’s behavior, mainly expressed in the several unsuccessful attempts to extract blood from the dolphin’s tail fin.

On 22.07.2017 dramatic changes were observed in the behavior of “Kako” dolphin. It was occasionally descended on the bottom of the pool, and showed very weak breathing activity during the water surfacing. Blood analysis revealed growing number of WBC’s (17.19 k/mkl), also sharply increased erythrocyte sedimentation rate (ESR) (54 mm / hr), and reduced hemoglobin value (12.3 g / dl). Because of sensitive behavioral changes and dysfunctional hematological indicators the antibiotic Bactamide (ampicillin sulbactam) was administered intramuscularly (2g IM SID) along with Rifampicin capsules (3 capsules 2.5 mg / kg PO BID) and 2 g of Lymphomys, a homeopathic remedy (Biologische Heilmittel Heel, Germany) for improvement of lymphatic circulation. The repeated test of “Kako” dolphin’s exhaled air band blood analyses have been done.

On 24.07.2017 in the first half of the day, the “Kako” dolphin was active, even became involved in coaching and in the exercise sessions. In the second half of the day, during the exercise session (16:30pm) the sudden dramatic changes of the animal’s health status was manifested in the breach of the coordination of the animal, started convulsions continued for 3-4 minutes. The animal was rapidly transferred to the pool’s pedestal and immediately was subjected immediately to emergency reanimation, in particular, injection into dorsal muscle 4 ml of cordiamine, 2 ml sulfocamphocain, 4 ml dexamethazone and 2ml of adrenaline, but despite of the actions it appeared not possible to save the animal. The extensive foam was discharged from the respiratory hole and the animal died.

Report on Necropsy and histopathological analysis:-
Two hours later “Kako” dolphin’s death of the dolphinarium’s veterinary service conducted the autopsy of the dead animal according to the international protocols aiming macroscopic examination of the internal organs

| ALP | U/l  | 300-1300 | 166 | 190 | 206 | 220 | 186 | 125 | 65 |
|-----|------|----------|-----|-----|-----|-----|-----|-----|----|
| CK  | U/L  | 100-250  | 125 | 121 | 123 | 125 |     |     |    |
| TP  | g/dl | 6.0-7.8  | 7.5 | 7.2 | 7.9 | 7.9 | 7.9 | 8.1 | 9.8|
| ALB | g/dl | 4.3-5.7  | 4.7 | 4.6 | 4.9 | 4.9 | 4.7 | 3.8 | 3.9|
| GLOB| g/dl | 1.3-2.5  | 2.8 | 2.6 | 3   | 3   | 3.2 | 4.3 | 5.7|
| BIL-T| mg/dl| 0.1-0.2  | 0.6 | 0.4 | 0.5 | 1.1 | 1.2 | 0.8 | 0.5|
| Glucose| mg/dl| 90-170  | 80  | 75  | 67  | 42  | 53  | 55  | 62 |
| CREA| mg/dl | 1.0-2.0  | 1.2 | 1.1 | 0.9 | 1.2 | 1.7 | 1.6 | 1.7|
| BUN| mg/dl | 42-58   | 51  | 48  | 51  | 50  | 51  | 48  | 39 |
| K | mmol/L | 3.2-4.2  | 4.7 | 4.4 | 4.3 | 4.9 | 3.8 | 3.9 | 4.5|
| Ca-T| mg/dl | 8.5-10.0 | 9.3 | 9.8 | 10.4| 8.9 | 10.3| 10.6| 10.1|
| Na | mmol/L | 153-158 | 149 | 146 | 148 | 152 | 147 | 146 | 144|
| Mg | mg/dl | 1.7     | 1.8 |     |     |     | 2   |     | 1.7|
| Phos| mg/dl | 4.0-6.0  | 5.4 | 5.2 | 5.3 | 5.3 | 5   | 5   | 6.3|
of the dead animal and collecting samples of internal organs for the bacteriological and histological investigation (Rowles et al., 2001).

The approximate age of dead “Kako” dolphin was estimated as 13 years old. The animal’s size was 246 cm in length, weight- 165kg. During the autopsy, there was no significant visual changes in the internal organs, except for the heart. On the left ventricle there was a 4-5 cm diameter white insert which after the section was found to be an abscess filled with the yellowish-colored creamy-caseous mass. According to the macroscopic examination the preliminary cause of “Kako” dolphin’s death was determined as focal purulent myocarditis.

Samples were taken from internal organs (stomach, heart, lungs, kidneys, testes, large intestines, spleen and liver) for histopathology and swabs were taken from the heart, liver and lungs for bacteriological examination. The study aimed to determine the most probable cause of “Kako” dolphin’s death, based on preliminary report on macroscopic examination of the dead animal and the findings of the histopathological examination after necropsy. According to the commonly recognized experience (Freudee, 1990), the results of the histopathological analysis are the leading criteria in the determination of a cause of death.

**Figure 1**: The dead dolphin “Kako” before the autopsy.

**Fig 2**: The necropsy report on “Kako” Dolphin’s death: A) heart; B) heart section.
The arrows indicate the purulent mass in the heart section and site taking the material for histopathology and bacteriological analysis.

**Histopathological study:**
Tissue samples from the brain, stomach, heart, lung, kidney, testis, colon, spleen and liver obtained from the dead animal- dolphin “Kako” were fixed in 4% phosphate-buffered formalin, embedded in paraffin, cut into 4 μm sections, stained with hematoxylin and eosin (H&E) and Periodic Schiff Acid (PAS) and examined in the light microscope.

Main histopathologic findings were extensive fibrosis with pyo-granulomatous lesions in the heart. In the adjacent myocardium fibroangioblastic tissue with scattered polymorph nucleated granulocytes and macrophages was present. Locally fibrin depositions on the epicardium with hemorrhage in the epicardial fat and thrombosis of an artery. Few macrophages in the tunica media of another artery. Periodic acid–Schiff (PAS) staining was negative for mycotic elements. The pleura of the lungs showed fibrosis with mild non-suppurative infiltrates. Pulmonary interstitial lymphoplasmocytic and neutrophilic infiltrates with edema and intra-alveolar macrophages was present. The multiple infiltrations in the lungs possibly indicated the primary route of infection.

![Figure 3](image-url)

**Figure 3:**- Histopathological study of the heart tissue of dead “Kako” dolphin. Microscopy picture of the thin section of heart tissue sample. Staining with hematoxylin, eosin and PAS (Microscope Leica, Germany), magnification X200.

The results histopathological examination of tissue samples of dead dolphin “Kako” lead to a strong assumption about the infectious cause of the death, namely the infections of the heart- myocarditis. For the confirmation of the preliminary conclusion on the primary cause of dolphin’s death the bacteriological investigation of the necropsy material has been done.

**Bacteriological analysis of pathological material:**
Bacteriological smears from dead dolphin’s organs, primarily heart, lung and liver tissues have been sampled with sterile swabs following a small section done by sterile instruments. The swabs were placed into the tubes with Tryptic Soy Broth (TSB) and after incubation 24h at 37°C the content was streaked onto Tryptic Soy Agar (TSA) with 5% sheep blood. Developed colonies after overnight incubation at 37°C, including those with β-hemolytic activity, were recorded.

It should be noted that from the liver and lung tissue samples monocultures were grown. The lung’s sample was represented by β- hemolytic, mucoid, 3-4mm in size gray colonies. In case of liver tissue sample cream - yellow, 2-2.5mm -sized colonies were developed. From heart sample mixed culture was isolated- one isolate was β-hemolytic, 2-3 mm diameter yellowish colonies and another -γ- hemolytic white- cream colonies with D=2-3 mm.
The further characterization of 4 selected isolates (1 from liver, 1 lung and 2 different isolates from the heart) was done. Gram staining showed that the both isolates from heart (KH1 and KH2) were G+cocci with grape like arrangement. Bacterial isolates from liver and lung (KL3 and KL4) appeared to be G- rod-shaped bacteria. For identification of selected isolates several basic biochemical tests were performed: KOH, Citochrom oxidase, catalase, amino acid utilization and carbohydrate fermentation tests. Also API 20E ,API 20 NE and APIStaph test systems (Biomereux, France) were used.

The both isolates from the heart – KH1 and KH2 were attributed to Staphylococcus spp. One of them (KH1) was biochemically (API STAPH) identified as S. aureus although with some atypical traits such as weak mannitol fermentation. The second isolate from heart- KH2 appeared to be also a coagulase negative Staphylococcus, biochemically identified as S. xylosus, which can cause opportunistic infections in humans. In general, as mentioned above, staphylococci, especially S. aureus, are considered as high risk pathogens for marine mammals, that my lead to serious complications, even fatal outcome (Streitfeld & Chapman, 1976).

The liver isolate KL3 was identified as nonfermenting bacterium Spingomonas paucimobilis, which is known as opportunistic human pathogen, mainly causing nosocomial infections. By our knowledge this is the first report of isolation of S. paucimobilis from dolphin’s tissues. The KL4 isolate, obtained from “Kako” dolphin’s lung tissue sample was identified as E. coli characterized with inability to ferment lactose and sorbitol, attributing this strain to pathogenic varieties of E.coli (serotyping hasn’t been done).

The susceptibility of the obtained bacterial isolates to antimicrobial drugs was studied by Kirby-Bauer disk-diffusion method. Heart tissue isolates – staphylococci showed susceptibility to the most of the 20 tested antibiotics, including cephalosporins of different generations. Resistance has been shown only to glycopeptide-vancomycin and 3rd generation cephalosporin-cephazidime, also to erythromycin in the case of KH2 isolate. It is noteworthy that erythromycin was involved in a treatment scheme for some time, which could have contributed to the development of resistance to this antibiotic. The both staphylococcal isolates appeared to be resistant to vancomycin - the antibiotic which is considered as a last resort drug for serious gram-positive infections, and in particular, for the MRSA treatment.

The strain E.coli KL4, isolated from lung tissue showed much higher resistance – it appeared to be unsusceptible to 9 tested antibiotics, therefore it could be considered as a multi drug-resistant bacterial isolate. The resistance was noted for the beta-lactam group antibiotics, macrolides, tetracycline, carbapenem and quinolones. Ciprofloxacin and erythromycin, as well as ampicillin and amoxicillin, were used in the treatment scheme of dolphin “Kako”, thus resistance may be due to the selective press. On the other hand, if the bacteria with such resistance persisted in the “Kako” dolphin’s organism, this could be one of the possible explanations of the unsuccessful antibacterial treatment, especially if we assume the initial infectious process in the lungs.

The two staphylococcal isolates from the dolphin’s heart – S. aureus KH1 and KH2 and lung isolate KL3 have been tested also for susceptibility to bacteriophages: commercial preparations “Staphylococcal bacteriophage”, “Pyophage”, “Intestiphage”, “Enco”, “Fersis” and “SES” (“Eliava Biopreparations”, Georgia) and individual phages from the Lab collection of the Eliava Institute. Both S.aureus KH1 and S. xylosus KH2 showed susceptibility only to phage Sa92, while E. coli KL3 was lysed by “Pyophage” and “Intestiphage” preparations, and individual phages –DDVI, Un and T4, that could indicate good chances for sick animal if alternative treatment- phage therapy would be used.

Discussion:-

The results of the necropsy, namely macroscopic examination of different organs of the dead animal along with histopathological findings (see above) allowed to categorize the death of dolphin “Kako” as of infectious origin, in particular, as infection of multiple organs with a purulent infection of the heart (infection of the myocardium) as a leading organ. According to the commonly accepted practice and opinion the pathologic examination remains the primary basis for determination the cause of death (Froede, 1990). In many histopathological studies on adult marine mammals bacterial infection(s) were found to be the most probable cause of the mortality (McFee and Lipscomb, 2009). The results of the necropsy and histopathological findings for the Dolphin “Kako” were also in line with the blood parameters obtained before the death of animal, also bacteriological investigation of necropsy material, yielded in the growth of bacteria(S. aureus, S. xylosus, E. coli), capable of causing inflammatory-purulent processes in internal organs of the animal.
The fact that the illness hasn’t been manifested during several months as abnormal behavioral or bad mood can be also not surprising, because marine mammals are known to hide their weakness for their safety and self-confidence in the community (Dunn et al., 2001). Bacteriology of Dolphin’s exhaled air samples done during up to 5 months observational period often showed extensive bacterial growth while majority of parameters stayed within normal range but the change in the WBC counts and neutrophilosis, only at the final stage the ESR was increased significantly. Such situation also was described by other authors (Liong et al., 1985) indicated that in the process of illness the hematological values were in the normal range, with only slight change of WBC. Another reason for the keeping blood parameters in considerably normal range could be the continuous treatment with anti-infectious and immunomodulatory preparations, that finally turned out to be unsuccessful and didn’t save the life the animal.

References:
1. Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J ClinPathol 45, 493–496.
2. Bossart G. Thomas H, Reidarson, Leslie A. Doerauf and Deborah A. Diffied. Clinical Pathology/ in CRC Handbook of Marine Mammal Medicine: Health, Disease, and Rehabilitation, Second Edition, Edited by Leslie Dierau, Frances M.D. Gulland, June 27, 2001 by CRC Press, pp.383-436.
3. Chieh Lo, Wen-Ta Li; I-Fan Jen. Group B Streptococcus (GBS) Infection in a Captive Bottlenose Dolphin (Tursiops truncatus gilli) Newborn Calf. IAAAM meeting and conference, 2015 Chicago, USA, April 10-15, 2015., http://www.vin.com/apputil/content/defaultadv1.aspx?pld=12676&meta=Generic&id=6651324
4. Dunn L.J, J. D.Buck, and T.R. Robeck. Bacterial Diseases of Ceataceans and Pinnipeds/ in CRC Handbook of Marine Mammal Medicine: Health, Disease, and Rehabilitation, Second Edition, Edited by Leslie Dierau, Frances M.D. Gulland, June 27, 2001 by CRC Press, pp.309-336.
5. Froede R.C. Handbook of forensic pathology. 2009. College of American Pathologist, Northfield, Illinois, pp.9–37.
6. Howard E.B, Britt JO, Matsumoto G.K, Itahara R, Nagano C.N (1983) Bacterial diseases. In: Howard EB (ed) Pathobiology of marine mammal diseases, Vol. 1. CRC Press, Boca Raton, FL, p 69–118.
7. Kropinski A., A.Mazzacco, T. Waddel E, Lingohr R and R. Jonson: “Enuomeration of Bacteriophages by Double Agar Overlay Plaque Assay”. In: Bacteriophages, Methods and Protocols”, Vol. 1. Isolation, characterization and interactions” (Ed. M.R.J. Clokie, A.M. Kropinski) 2009, Humana Press, Chapter 7, pp 69-80.
8. Liong E., Dd. Hammond and N.A. Vedros. Pseudomonas pseudomallei infection in a dolphin (Tursiops gilli): a case study. Aquatic mammals, 1985, v.1, pp. 20-22.
9. McFee E.W. and Lipscomb P.T. Major pathological findings and probable causes of mortality in bottlenose dolphins stranded in South Carolina from 1993 to 2006. Journal of Wildlife Diseases, 2009, 45 (3) 2009, pp.575-593.
10. Miller W.G, Adams L.C, Ficht T.A, Cheville N.F, Payeur J.P., Harley, D.R., House, C., and Ridgway S.H. Brucella-induced abortions and infection in bottlenose dolphins (Tursiopstruncatus). Journal of Zoo and Wildlife Medicine, 1999, 30: 100-110.
11. Palmer C.J., J. P. Schroeder, R. S. Fujioka and J. T. Douglas. Staphylococcus aureus Infection in Newly Captured Pacific Bottlenose Dolphins (Tursiops truncatus gilli). Journal of Zoo and Wildlife Medicine, 1991, v. 22, No. 3, pp. 330-338.
12. Romanov V.V., M. B. Chelysheva. Hepatopathy in a Captive Black Sea Bottlenose Dolphin with Mixed Bacterial and Fungal Infection: Case Report. IAAAM conference Proceedings, 2009. http://www.vin.com/apputil/content/defaultadv1.aspx?pld=11285&meta=Generic&id=3976353
13. Rowles T.K., F. M. Van Dolah and A. A. Hohn, Gross Necropsy and Specimen Collection Protocols/ in CRC Handbook of Marine Mammal Medicine: Health, Disease, and Rehabilitation, Second Edition (Ed. Leslie Dierau, Frances M.D. Gulland), 2001, CRC Press, pp. 449-470.
14. Schaefer AM, Goldstein GD, Reif JS, Fair Pa, Bossart GD. Antibiotic-resistant organisms cultured form Atlantic bottlenosedolphins (Tursiops truncatus) inhabiting estuarine waters of Charleston, SC and Indian river lagoon, FL. Ecohealth, 2009, v.691, pp.33-41.
15. Streifeld MM, Chapman CG. Staphylococcus aureus infections of captive dolphins (Tursiops truncatus) and oceanarium personnel. Am J Vet Res. 1976, 37(3):303-5.
16. Song Z., Yue R., Sun Y., Liu C., Khan Sh., Li C., Zhou X., Yang L., Zhao D. Fatal bacterial septicaemia in a bottlenose dolphin Tursios truncates casued vt Streptococcus iniae .Dis.aquat organ., 2017, 122 (3), pp.195 -203.
17. Stewart JR., Townsend FL., Lane SM., Dyar E, Hohn AA, Rowles TK, Staggs LA, Wells RS, Balmer BC, Schwanke LH. Survey of antibiotic –resistant bacteria isolated from bottlenose dolphins tursiops truncates in the southeastern USA. Ecohealth, 2009, v.6 (1), pp.33-41.

18. Tserodze T., N. Zobova, D. Jgenti, M. Mgeladze, R. Goradze, E. Jaiani, E. Didebulidze, and M. Tediaishvili. 2016. Study of Water Hydrochemical and Mikrobiological Quality in the Noogenic Habitat of the Black Sea Bottlenose Dolphins. International Journal of Advanced Research (IJAR), 4(9) ISSN: 2320-5407, pp.67-71.

19. Venn-Watson S., C. R. Smith, E.D. Jensen. Primary bacterial pathogens in bottlenose dolphins Tursiops truncatus: needles in haystacks of commensal and environmental microbes. Dis.aquat.org. 2008, V.79 (2), pp. 87-93.