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ISOLATION OF *STAPHYLOCOCCUS PASTEURI* IN THE CULTURED RUSSIAN STURGEON (*ACIPENSER GUELDENSTAEDTII*) IN BULGARIA

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ABSTRACT

The present study describes the first case of staphylococcosis causing *Staphylococcus pasteuri* in Russian sturgeon (*Acipenser gueldenstaedtii*) in Bulgaria. Clinically, diseased three sturgeons showed hemorrhagic ulcerative skin lesions at the all body surface and internally hyperemia and hemorrhages in the visceral organs and muscle, necrosis in the liver and swollen spleen were observed. Biochemical tests and Vitek 2 system were used to determine the phenotypic characteristics of isolated bacteria in samples taken from liver, spleen and kidney. The isolated bacteria were identified as *Staphylococcus* sp. Furthermore, 16S rRNA gene of one isolate was partially sequenced and showed 98% identity with the Genbank sequences of *Staphylococcus pasteuri*. The isolates were determined to be sensitive to sulfamethoxazole-trimethoprim, enrofloxacin and florfenicol, they were resistant to erythromycin, ampicillin and ciprofloxacin. Histopathologically, hemorrhage and lymphocyte groups in the epicardium; intense necrotic areas in the heart; melonomacrophage centers, lymphocyte infiltration around the necrotic hepatocyte cells; necrosis in the kidney; hyperemia and intense hemorrhagic areas in the spleen and melonomacrophage foci, hyperplasia and increase in the number of the goblet cells in the gills were observed. This study represents the first report of *S. pasteuri* isolation and identification as an agent of diseased Russian sturgeon.

Keywords: Russian sturgeon, *Staphylococcus pasteuri,* 16S rRNA gene, antibiogram, histopathology

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INTRODUCTION

In line with the findings of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) data analysis, China, France, Italy, the USA, Germany and Bulgaria were the most frequently found as countries of sturgeon production (Harris and Shiraishi, 2018). Sturgeon aquaculture is great economic and ecological importance for the Lower Danube region countries (Bulgaria, Romania, Serbia, Ukraine and Moldova) and has increased excessively in last decade (Smederevac-Lalić *et al.,* 2011; Rusenov *et al.,* 2019). Several sturgeon species of the *Acipenseridae* family are cultured in Bulgaria such as beluga (*Huso huso*), Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon (*Acipenser baerii*) and interspecific hybrids by artificial fertilisation. However, the Russian sturgeon is the most common species of sturgeon and as such is exposed to numerous diseases (Radosavljević *et al.,* 2019).

Bacterial infections, affecting cultured sturgeon include motile aeromonas septicemia, pseudomonadiasis, flavobacteriosis and staphylococcosis (Öztürk and Altınok, 2014; Xu *et al.,* 2015). In the recent years, it is increasingly recognized that the Gram-positive cocci, especially Staphylococcus species are important fish pathogens (Soliman *et al.,* 2014; Austin and Austin, 2016; Yilmaz *et al.,* 2019). Staphylococcus is a Gram positive, spherical, mostly coagulase positive bacterium belonging to the *Staphylococcaceae* family, order Bacillales (Gherardi *et al.,* 2018) and widespread in the nature (Čuvalová *et al.,* 2015). Staphylococci are reported as pathogens of the cultured sturgeon species (Rusev *et al.,* 2016; Kayiş *et al.,* 2017; Babaalian *et al.,* 2020). In fact, for one of the Staphylococci, *Staphylococcus pasteuri* described as a potential fish pathogen there is a lack of information in literatures with cultured sturgeons. The origin of *S. pasteuri* remains unknown; however, it has been identified in waste and drinking water (Mauriello *et al.,* 2004; Faria *et al.,* 2009; Čuvalová *et al.,* 2015) and isolated from the intestinal tract of cultured yellow seahorse (*Hippocampus kuda*) (Tanu *et al.,* 2012) and skin of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) (González *et al.,* 1999).

In the present study clinical signs, bacteriological, molecular and histopathological findings demonstrated that *S. pasteuri* caused mortality in the cultured Russian

sturgeon (*Acipenser gueldenstaedtii*) in Bulgaria. To our knowledge, this is the first report of the staphylococcosis caused by *S. pasteuri* in the cultured Russian sturgeon.

MATERIALS AND METHODS

Sample collection: Five years of age Russian sturgeon (n=3) (4.240±95.75g) showing signs of large ulcerative skin lesions and hemorrhages on the body surface were obtained from licensed sturgeon farm located on Southeast Bulgaria. Water temperature (16.3 C°), dissolved oxygen (9.8 $mg.L^{-1}$), NO3-N (0.9 $mg.L^{-1}$), and pH 7.1 were determined using portable multi meter (HQ40D, Hach Corp., US) at the same time of sampling (February 2019). In this season, a disease outbreak with 5 % fish losses occurred in the sturgeon farm.

Isolation and identification: Samples were taken from the liver, spleen and kidney from all diseased sturgeon. They were inoculated onto Blood Agar (BA), Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA). Petri plates were incubated during 24-48 h at 24-25 C°. The isolates recovered from the diseased sturgeon were identified by using the conventional bacteriological method and Vitek 2 system (Buller, 2004; Savini *et al.,* 2009; Austin and Austin, 2016).

Moleculer identification of a representative strain: A representative strain (MKc_{05}) of similar isolates was chosen for molecular analysis. One colony of MKc_{05} was transferred to a tube (2ml) containing Tryptic Soy Broth (TSB) and incubated during 24-48 h at 24-25 C° until the OD_{600} was 1. After incubation, 1.0 ml of the bacteria culture was centrifuged at 12,000 g for 1 min, the supernatant was discarded, and the pellet was frozen at −20˚C until DNA extraction. Genomic DNA was extracted using the Roche Genomic DNA Purification Kit (11796828001, Germany) according to the manufacturer's instructions. The extracted DNA from the MKc_{05} was subjected to PCR with the universal bacteria primer set (U8F (5' AGAGTTGATCATGGCTCAG 3'), 1492R (5'GGTTCACTTGTTACGACTT3') as reported by Weisburg *et al.* (1991). PCR products were sequenced bidirectional by Medsantek (Istanbul, Turkey).

Antibiotic susceptibility test: Antibacterial susceptibility of the isolates was determined by using Kirby Bauer disc diffusion method on Mueller-Hinton Agar (MHA) (Himedia-M173) (Barry and Thornsberry, 1985). For this reason, 12 commercial antibiotic disc such as erythromycin (5 µg/disc), ciprofloxacin (1 µg/disc), oxytetracycline (30 µg/disc), florfenicol (30 µg/disc), chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), flumequine (30 µg/disc), streptomycin (10 µg/disc), sulphamethoxazole (25 µg/disc), ampicillin (10 µg/disc), enrofloxacin (5 µg/disc) and furazolidone (50 µg/disc) (Oxoid, England) were used. The antibiotic sensitivity test was carried out according to instruction of the Clinical and Laboratory Standard Institute (CLSI, 2010), and performed in duplicates. The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R).

Histopathological examination: Tissue samples from visceral organs (liver, spleen, kidney, intestine, stomach, and heart), skin and gill immediately fixed in 10% buffered formalin and processed for paraffin embedding. Paraffin blocks were sectioned (4-5 µm) on a microtome Leica RM 2125 (Leica Microsystems GmbH, Austria), dewaxed and stained with haematoxylin (Sigma-Aldrich-HHS16), and eosin (Merck 109844, Germany) (H&E), according to the method described by Culling (1963).

RESULTS

Clinical findings: All diseased fish exhibited externally hemorrhagic ulcerative skin lesions at the all body surface especially ventral surface, hemorrhages on the base of the fins and around the anus (Fig. 1a). Internally hyperemia and hemorrhages in the visceral organs and muscles, necrosis in the liver (Fig. 1b) and splenomegaly were observed.

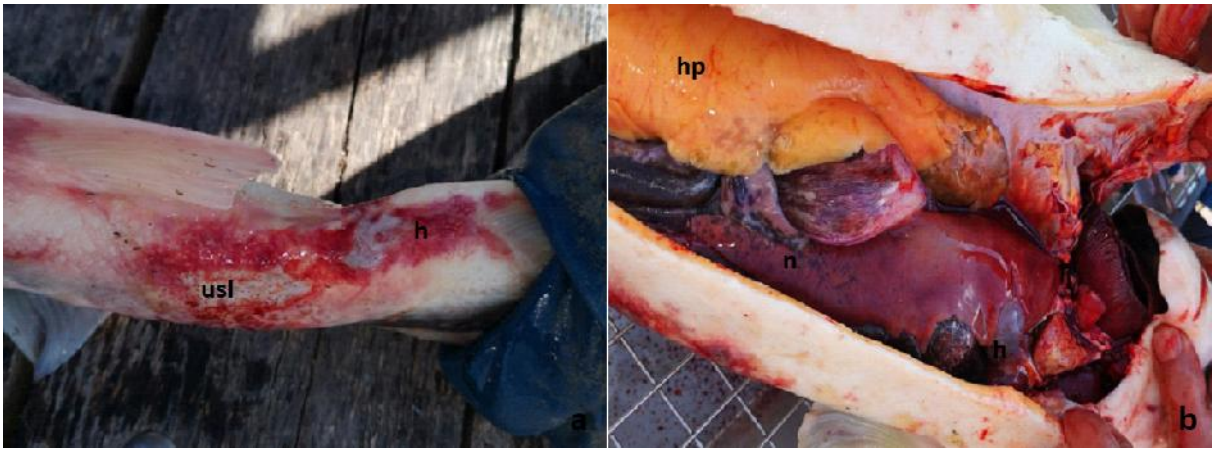

Figure 1. Diseased Russian sturgeon. (a): Intensive hemorrhages (h) and ulcerative large skin lesions (usl) on the lateral and ventral body surface. (b): Hemorrhages (h) hyperemia (hp) in the visceral organs and necrosis (n) in the liver

Bacteriological findings: After the incubation of the bacteriological inoculations from the visceral organs, raised with regular edges and light yellow pigmented, single bacterial colonies on TSA and BHIA were observed at 24-25 C° for 48 hours (Fig. 2). They (n=10) were non-motile, Gram-positive, clusters, facultative fermentative, cytochrome oxidase negative, catalase positive, resistance to bacitracin, grow at 15-45 C° and coagulase negative. Therefore, the isolates were identified as *Staphylococcus* sp.. The bacterium did not produce arginine dihydrolase, ornithine decarboxylase, not degrade aesculin. It produced acid from glucose, sucrose, maltose, trehalose and fructose but not from arabinose, lactose, xylose mannose.

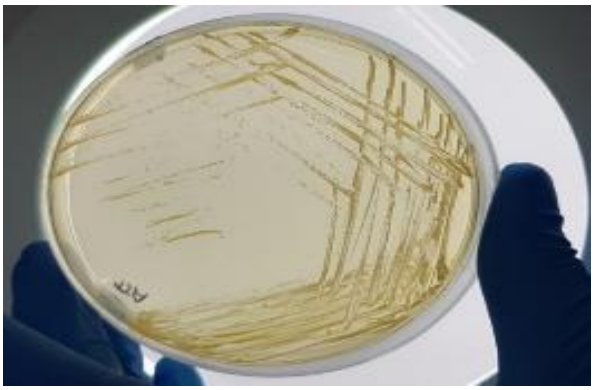

Figure 2. Yellow pigmented *Staphylococcus* sp. colonies isolated from diseased Russian sturgeons.

According to Vitek 2 analysis, isolates were identified as *S. warneri* (96%), but possibility of *S. pasteuri* were observed because of the isolates had yellow pigmented (Fig. 2). So this result was confirmed by using 16S rRNA gene sequence analysis. According to 16S rRNA gene sequence analysis of the representative isolate (MKc_{05}) was identified as *Staphylococcus pasteuri*. The sequence obtained in this study is defined as GenBank accession number MW307978.

Antimicrobial susceptibility test findings: All isolates were highly sensitive to enrofloxacin, florfenicol and sulfamethoxazole. They were resistance to ampicillin, ciprofloxacin, erythromycin and intermediate resistance to others chemotherapeutics (Table 1).

Table 1: Results of the antibiotic susceptibility test.

Antibiotic	Resistance	Inhibition Zone Diameter (mm)
Ampicillin (10 µg)	R	0<15
Chloramphenicol (30 µg)	I	15<20.0<25
Ciprofloxacin (1 µg)	R	0 <15
Enrofloxacin (5 µg)	S	30.5 >25
Erythromycin (5 µg)	R	0<15
Florfenicol (30 µg)	S	28.5 >25
Flumequine (30 µg)	I	15<17.5<25
Furazolidone (50 µg)	I	15<18.4<25
Kanamycin (30 µg)	I	15<17.0<25
Oxytetracycline (30 µg)	I	15<19.0<25
Streptomycin (10 µg)	I	15<19.0<25
Sulfamethoxazole (25 µg)	S	31.5 >25

(R: resistant; **I:** intermediate; **S:** sensitive)

Histopathological findings: Histopathologically; hemorrhage and lymphocyte groups in the epicardium (Fig. 3a), intense necrotic areas in the ventricle (Fig. 3b); exudate accumulation (Fig. 4a), melonomacrophage groups (Fig. 4b), lymphocyte infiltration in the liver (Fig. 4c), swelling in the nuclei and necrosis in the hepatocyte cells especially intense necrosis of the hepatocyte cells on the outer surface of the liver and hyperemic areas were observed (Fig. 4d). Also necrotic areas in the anterior kidney (Fig. 5a); hyperemia and intense hemorrhagic areas in the spleen (Figure 5b); hyperplasia in the secondary gill lamellae, increase in the number of goblet cells and melonomacrophage foci in the gill (Fig. 6a); hyperemia and infiltration of inflammatory cells in the dermis layer of the skin and necrosis in muscle cells (Fig 6b) were noted.

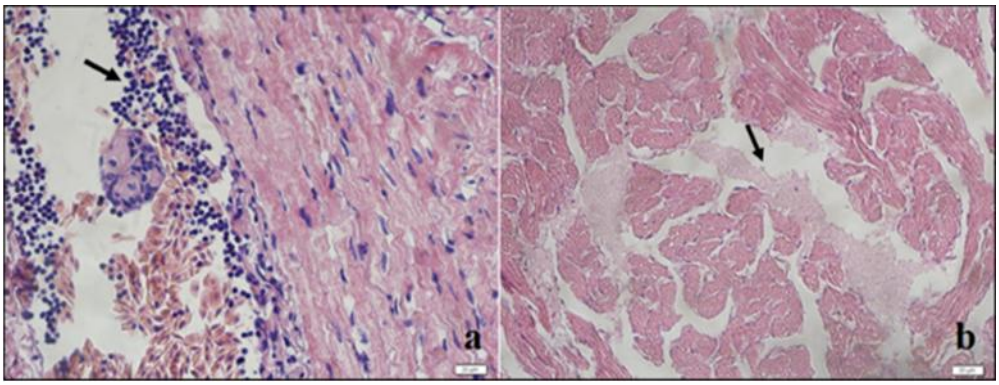

Figure 3. Hemorrhage and lymphocyte infiltration in the heart (arrowed) (a) (H&E stain; x40) and intense necrotic areas in the ventricle (arrowed) (b) (H&E stain; x20)

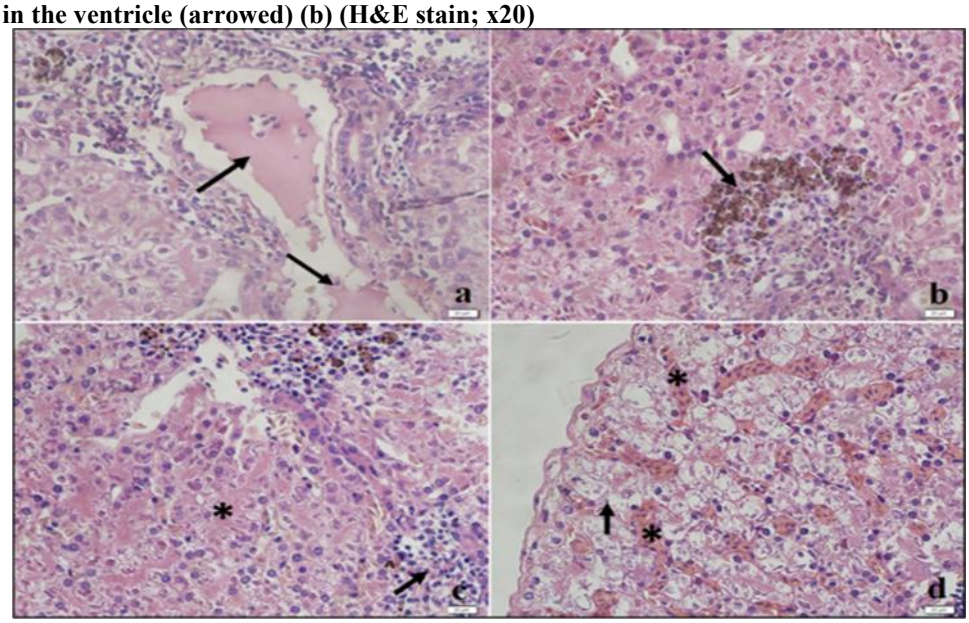

Figure 4. Exudate accumulation (arrowed) (a), melonomacrophage center (b), lymphocyte infiltration (arrowed), swelling in the nuclei and necrosis in the hepatocyte cells (*) (c), hyperemic areas (*) among the intense necrotic hepatocyte (arrowed) (d) (H&E stain; x40)

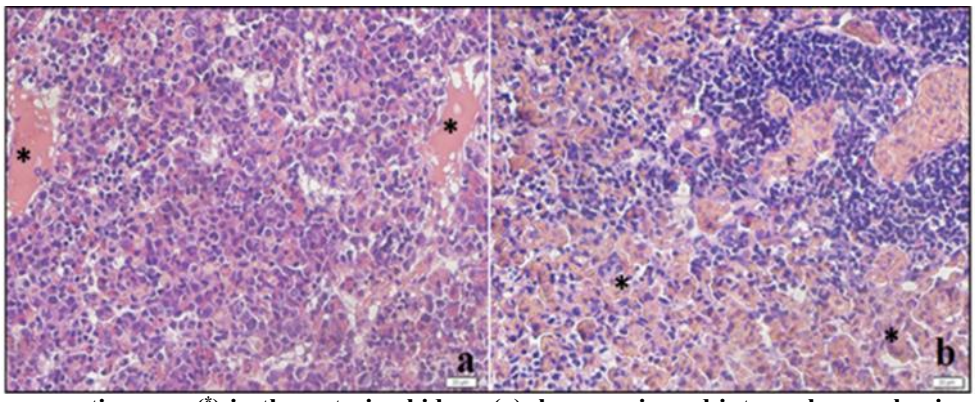

Figure 5. Large necrotic areas (*) in the anterior kidney (a), hyperemia and intense hemorrhagic areas (*) in the spleen (b) (H&E stain; x40).

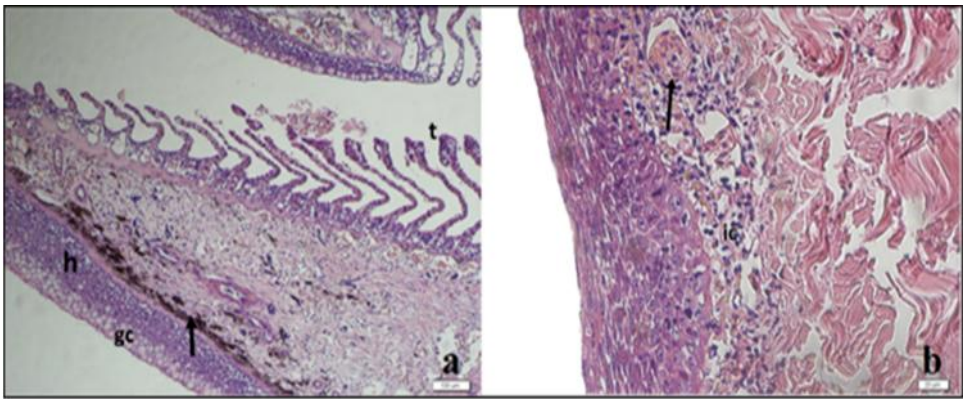

Figure 6. Telangiectasias (t) and hyperplasia (h) in the secondary gill lamellae, increase in the number of goblet cells (gc) and melonomacrophage foci (arrowed) in the gill (a) (H&E stain; x20), hyperemia (arrowed) and

infiltration of inflammatory cells (ic) in the dermis layer of the skin and necrosis in muscle cells (b) (H&E stain; x40)

DISCUSSION

Contrary to popular belief, the bacterial infections in the aquaculture sector are caused generally by Gram-negative bacteria; recent studies have reported that Gram-positive bacteria also cause infection in culture fish (Öztürk and Altınok, 2014; Rusev *et al.,* 2016; Kayiş *et al.,* 2017; Babaalian *et al.,* 2020). Although the most common primary pathogens of staphylococcosis such as *Staphylococcus epidermidis* and *Staphylococcus aureus* have been frequently reported from diseased fish (Kusuda and Sugiyama, 1981; Shah and Tyagi, 1986; Wang *et al.,* 1996; Kubilay and Ulukoy, 2004); in the recent years, a new species such as *Staphylococcus warneri* (Gill *et al.,* 2000; Metin *et al.,* 2014), *Staphylococcus hominis* (Yilmaz *et al.,* 2019), *Staphylococcus xylosus* (Oh *et al.,* 2019), *Staphylococcus capitis* (Yiagnisis and Athanassopoulou, 2011), and *Staphylococcus cohnii subsp. cohnii* (Akaylı *et al.,* 2011) have been reported in the different fish species.

Staphylococci are very common in nature and are part of the normal flora of the skin and mucous membranes. In additional, the zoonotic potential of them increase interest in their transmission mechanism via food, livestock, as well as domestic and wild animals (Fryer and John, 1993; Tenover and Gorwitz, 2006; Oh *et al*., 2019).

The clinical signs of staphylococcosis in fish are not specific. The most common clinical symptoms such as exophthalmia, loss and degeneration of the eye have been reported from different researchers (Shah and Tyagi, 1986; Oh *et al.,* 2019). In the current study, although no clinical findings were detected in the eyes of diseased Russian sturgeons, hemorrhages in the visceral organs reported by other researchers were determined (Wang *et al.,* 1996; Kubilay and Ulukoy, 2004; Yiagnisis and Athanassopoulou, 2011; Yilmaz *et al.,* 2019; Canak and Timur, 2020). In contrast to the results described by Kusuda and Sugiyama (1981) for ulceration on the tail in carp; Huang *et al* (1999) reported lesions in the epidermis and fin of tilapia (*Oreochromis* spp.) infected with *S. epidermidis*. In present study we observed similar that ulcerative skin lesions were observed on the lateral and ventral body surfaces of diseased sturgeons.

Isolates obtained from diseased Russian sturgeon were Gram-positive, cluster, non-motile, cytochrome oxidase negative and catalase positive, they were identified as *Stapylococcus* sp. Comparing our bacteriological findings with prior studies, reveals the similar to the biochemical results of *S. pasteuri* (Chesneau *et al.,* 1993; Savini *et al.,* 2009). It has been reported that *S. pasteuri* is phenotypically closely related to *S. warneri* and *S. hominis* (Savini *et al.,* 2009; Askarian *et al.,* 2012), but isolates obtained from diseased sturgeon were identified as *S. pasteuri* by using Vitek system according to the criteria previously described by Barbieri *et al.* (2005). These results were checked and confirmed by sequencing of 16S rRNA. The sequences obtained in this study have been deposited to GenBank and defined under accession number MW307978.

According to results of the disk diffusion method, all staphylococcal isolates exibited resistant to erythromycin, ampicillin and ciprofloxacin but sensitive to sulfamethoxazole, enrofloxacin and florfenicol. Similarly to our results, Regecová *et al*. (2016) confirmed the most frequent resistance to members of penicillin family, such as penicillin, ampicillin and oxacillin.

We observed the same focal necrosis in the hematopoietic tissue as described in previous histopathologically study in tilapia (*Oreochromis* spp.) infected with *S. epidermidis* except diffuse granulomas with necrotic centers (Huang *et al.,* 1999). In contrast to the results described by Rusev *et al.* (2016) for sturgeon infected with *S. warneri* and *Shewanella putrefaciens* have pycnosis and caryolysis in nuclei of the hepatic cells in the liver in current study swelling in the nuclei was observed. Especially, the most severe pathological reactions were noted in the liver such as melonomacrophage groups, lymphocyte infiltration, necrosis and hyperemia reported by Gaafar *et al.* (2015).

In conclusion, as far as we know, this is the first report on the isolation and identification of *Staphylococcus pasterius* in the diseased Russian sturgeon in Bulgaria. The antibiotic resistance test showed suitable antibiotics for use, in combination with low virulence suggest targeted antibiotic therapy whenever possible. Taking into account that *S. pasteuri* is member of Coagulase-negative staphylococci (CoNS) group and can become a potential pathogen for fish, it is necessary to characterize the role of virulence factor. Therefore, it would be essential to do further investigation on pathogenesis and prevention of *S. pasteuri* in cultured fish.

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