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# *Streptococcus iniae*: A Growing Threat and Causative Agent of Disease Outbreak in Farmed Chinese Sturgeon (*Acipenser sinensis*)

**Murtala Muhammad, Ting Zhang, Siyu Gong, Jing Bai, Jiansong Ju, Baohua Zhao* and Dong Liu***

*College of Life Science, Hebei Normal University, Shijiazhuang 050024, China*

## ABSTRACT

*Streptococcus iniae* infection of cultured fish species is in an alarming trend, due to the possible emergence of antibiotic resistant strains. The bacteria was responsible for the disease outbreak that caused massive mortality of Chinese sturgeon (*Acipenser sinensis*) in 2016. We isolated the pathogenic bacteria (HNM-1) from the infected *A. sinensis* and identified its identity by conventional physiological, biochemical, and molecular techniques. Virulence and pathogenesis of the disease were determined by intraperitoneal injection of the etiological agent to the healthy *A. sinensis*. Physiological and 16s rRNA molecular analysis identified *Streptococcus iniae* as the causative agent of the disease outbreak. The bacteria has a unique biochemical profile compared with most of the previously isolated strains. Antimicrobial susceptibility tests revealed that *S. iniae* HNM-1 is resistant to most of the important antibiotics, including Kanamycin, Amoxicillin, gentamycin, Penicillin G and spectinomycin. The bacteria is pathogenic to Chinese sturgeon via intraperitoneal injection. It instigated pathological changes in vital organs that lead to mortality of the infected *A. sinensis* with a cumulative mortality of 40% to 100%. *S.iniae* HNM-1 ability to evade the innate immune system was determined by whole blood killing, exposure to hydrogen peroxide, and biofilm assays. This study represents the first Report of *S. iniae* infection of Chinese sturgeon (*Acipenser sinensis*).

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**Authors' Contribution**
**BZ and DL conceived the project. ALL statistically analyzed the data and wrote the article. MM, JB, TZ, and SG executed the experimental work. DL, BZ and JJ supervised the research project.**

**Key words**
***Acipenser sinensis, Streptococcus iniae,* Virulence, Antimicrobial Resistance, Biofilm**

## INTRODUCTION

Sturgeon is the common name for the 27 species of fish belonging to the family Acipenseridae, which includes a Chinese sturgeon (*Acipenser sinensis)*; an essential delicacy in China, that was listed among critically endangered fish species. As such government banned its commercial fishing at its only natural breeding grounds (channel of the Yangtze River, the Pearl River, and the East and South China Seas). Which leads to an increase of more sturgeon artificial breeding farms across China (Zhang *et al*. 2012). However, the rapid increase of cultured fish farming and high stock density have resulted in higher susceptibility of sturgeon species to infections of bacteria such as *Aeromonas hydrophila, Aeromonas veronii* (Di *et al*., 2018), and *Streptococcus dysgalactiae* (Yang and Li, 2009).

*Streptococcus iniae* is a fish pathogen, which can cause a high mortality of about 50% to 75% of susceptible cultured fish. It has so far infected more than 30 fish species, significantly contributed to the economic losses of aquaculture industries across the world (Agnew and Barnes, 2007). The bacteria was initially isolated in 1976 from an Amazon freshwater dolphin (*Inia geoffrensis*) in San Francisco, California, USA (Pier and Madin, 1976). Subsequently, reports of mass mortality associated with *S. iniae* of both fresh, salt and brackish water fish species were documented (Kayansamruaj *et al*., 2017). The zoonotic bacteria was also reported to cause bacteremia, cellulitis, meningitis, and osteomyelitis in human (Lau *et al*., 2003). Not all *S. iniae* are virulent due to the lack of some critical virulent factors in some strains (Weinstein *et al*., 1997).

The primary objectives of this study, are to identify and characterize the etiological agent of the disease outbreak in Chinese sturgeon fish farm located at Hebei province of China during the summer of 2016, evaluate its virulence and pathogenesis in experimentally infected *A. sinensis,* as well as to determine the effective antibiotics treatment.

## MATERIALS AND METHODS

*Sampling*

Fish that showed clinical sign and symptoms of the disease were collected during the outbreak and sent to

* Corresponding author: pqw1234@163.com; baohua519@126.com
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the laboratory for further examinations. Liver, brain and spleen samples from the infected fish were aerobically inoculated on Brain heart infusion (BHI) agar plates and incubated at 30°C for 36 hours, followed by re-culture of a single colony on another BHI agar plates, then in BHI medium and incubated at 30°C for 24 hours. 20 % (V/V) of glycerol was added to the stock culture and stored at -80°C.

*Experimental animal*

Chinese Sturgeon (*A. sinensis*, 100-130 g) obtained from Hebei aquaculture experimental station, Shijiazhuang China, were grouped into four, each with 10 fishes and kept in recirculation freshwater tanks for experimental infection.

All fish experimental procedures were strictly carried out according to the recommendations in the Guide for the Care and Use of the Laboratory Animals of Hebei Province of China. The animal experiment protocol was approved by the Animal Monitoring Committee of Hebei Normal University.

*Phenotypic and biochemical characterizations of the isolate*

The isolate was cultured on Tryptone soy yeast extract agar (TSYEA) (Sigma-Aldrich) plates, incubated at 30°C for 24 h, and used for gram stain and capsule stain, according to standard protocols. While hemolysis was determined using Tryptone soy agar plates supplemented with 5% sheep blood (Henan Zhengzhou, China). Optimum growth temperature and salt tolerance test were conducted by incubating the bacterial culture at different temperatures (10°C, 20°C, 30°C, 35°C and 45°C) and also in TSYE medium containing different salt concentrations (0%, 1.0%, 3.0%, 5.0% and 6.5%) respectively. Biochemical profile of the isolate was determined by using the API-20 strep kit (Biomerieux, France) according to the manufacturer's instructions.

*Molecular identification and phylogenetic analysis*

Genomic DNA of the isolate was extracted using the TIANamp Bacterial DNA kit (Tiangen Biotech, Beijing China). PCR amplification was performed using species-specific PCR primers targeting genes of *16S rRNA* (F - 5'-AGAGTTTGATCCTGGCTC-3' and R-5'-TCAAAGGCTGCTGGAC-3') (Jensen *et al*., 2002) and lactate oxidase (*Lox*) (F- 5'-GAAATGATAAATGCGACTAC-3' and R- 5'-TCAAAGGCTGCTGGAC-3') (Mata *et al*., 2004). DNA sequencing analysis was conducted using the ABI 3730x1 DNA sequencer (Applied Biosystems). Genetic distance tree analysis of *16S rRNA* gene was build using MEGA 5.0 software.

*Antimicrobial susceptibility test*

The sensitivity of the isolated strain to 19 kinds of antimicrobial agents was determined by disc diffusion method on TSYEA plates, using antimicrobial disks (Hangzhou Tai he, Hangzhou China), according to the Clinical and laboratory standards institute (CLSI-2015) guidelines.

*Virulence genes of* Streptococcus iniae

Six essential virulence genes of *S. iniae* were detected by PCR, as described previously with little modifications (Deng *et al*., 2017). Primers sequences and gene descriptions were offered in Table I.

**Table I. Primers used for the amplification and sequencing of virulence genes, virulence genes of the bacteria were detected by using the listed primers; the PCR products were sequenced and confirmed by blast analysis.**

| Primer set | Primer | Sequence (5' to 3') | Virulence factors | Amplicon Size (bp) |
|---|---|---|---|---|
| A | *scpI*-a | GCAACGGGTTGT CAAAAATC | C5 α peptidase | 822 |
| | *scpI*-b | GAGCAAAAGGAGTTGCTTGG | | |
| B | *simA*-a | CGCAAAATGATCACATCAGCAGTCCTTG | M-like protein | 760 |
| | *simA*-b | CAGCTCCAACCATAACCGCGATAGCAC | | |
| C | *Pdi*-a | TTTCGACGACAGCATGATTG | Polysaccharide | 381 |
| | *Pdi*-b | GCTAGCAAGGCCTTCATTTG | Deacetylase | |
| D | *saga*-a | AGGAGGTAAGCGTTATGTTAC | Cytolysin SLS | 190 |
| | *saga*-b | AAGAAGTGAATTACTTTGG | | |
| E | *Pgm*-a | ATGACTTATACAGAAAATTATCAAAAATG | Phosphoglucomutase | 1700 |
| | *Pgm*-b | ACAAAAGTGTTGATTTCAGCTTCAATTG | | |
| F | *cpsD*-a | TGGTGAAGGAAAGTCAACCAC | Capsule | 534 |
| | *cpsD*-b | TCTCCGTAGGAACCGTAAGC | | |

*Scanning electron microscope*

The ability of *S. iniae* to produce biofilm was analyzed using a scanning electron microscope (SEM); Conducting glass and 1ml of HNM-1 culture were added to the wells of 24 wells plate, and incubated at 35°C for 6 days. The glasses were washed with 0.1M PBS and fixed for 6 hours in 2.5% buffered glutaraldehyde, washed again in 0.1M PBS, and dehydrated in a graded series of ethanol solutions (50-100%), After critical point drying, the specimens were mounted on SEM discs, coated with gold and observed with a Hitachi S-570 scanning electron microscope (Hitachi, Japan) at an accelerating voltage of 60 kV (Liu *et al*., 2015).

*Reactive oxygen assays*

The isolated strain was sub-cultured to OD (600 nm) of 0.5 and incubated for 2 hours in 1mM and 2mM concentrations of hydrogen peroxide ($H_2O_2$) (Sigma–Aldrich, St. Louis, MO) at $30^0$C, each in triplicate. One thousand U /mL of Catalase (Sigma–Aldrich, St. Louis, MO) was added to quench $H_2O_2$ at the end of the assay. Dilutions were plated on TSYEA plates to determine the number of surviving CFU, control samples were prepared by inoculating the bacteria without the addition of hydrogen peroxide. Percent survival was calculated as (CFUs after co-incubation in $H_2O_2$/ CFU in control inoculums) ×100 (Buchanan *et al*., 2008).

*Whole-blood killing assay*

100µl of diluted log-phase bacteria culture with OD (600 nm) of 0.5 was added to 300µl of fresh fish heparinized blood (sodium heparin, Sigma–Aldrich, St. Louis, MO) from three individual fish and incubated at 35°C in a shaker for 2.0 hours. The percentage of live bacteria was determined by plating on TSYEA and calculated as follows: (CFUs after co-incubation/ CFUs in initial inoculums) ×100% (Buchanan *et al*., 2008). The negative control was made by direct inoculation of the 100µl of PBS directly into the heparinized fish blood.

*In vivo infection assay*

Preliminary pathogen screening of fish was conducted to make sure they were in good health. HNM-1 culture, diluted to concentrations of approximately 2.0 x $10^5$, 2.0 x $10^6$, 2.0 x $10^7$ CFU were intraperitoneally injected to the healthy Chinese sturgeon, one concentration per group, while the control group was injected with sterile Phosphate buffered saline (PBS). Mortality was recorded every day for 7 days post-infection and only attributed to the inoculated bacteria when it was recovered from the dead fish in pure culture and confirmed by PCR using16S rRNA and *lox* Genes specific primers.

*Histopathological analysis*

The collected Liver, heart, brain and spleen samples of the fish were fixed in 10% buffered formalin (pH 7.2) for 24 h, embedded with paraffin Sections (4µm) and stained with Hematoxylin and Eosin (HandE) as well as examined by light microscopy (Nikon Eclipse ci, imaging system: NIKON digital sight DS-FI2, magnification: 200×, 400×).

*Statistical analysis*

Statistical analysis was performed with SPSS 16.0 (SPSS Inc. USA) and the Prism software program 7.0 (GraphPad Software, Inc. USA). Survival data were analyzed with the log-rank test. The *P* values in other experiments were obtained using Student's *t*-test. *P* values $< 0.05$ and $< 0.01$ were considered statistically significant and highly statistically significant, respectively.

## RESULTS

*Phenotypic and molecular identification of the isolate*

The isolated bacteria were identical in morphology, having a small round smooth, white colonies with a diameter of 0.8-1.0 mm, it is capsulated, β-hemolytic, gram-positive bacteria arranged in pairs, and chains of varying lengths, with the optimum growth temperature within the range of 30°C to 35°C, but unable to grow at 10°C and 45°C. Salt tolerance test results showed that the bacteria is not viable in a broth medium with 6.5% NaCl concentration but very slow in media with 5% NaCl for two days which subsequently able to tolerate the salt and grow viably.

Expected PCR products sizes of *16S rRNA* (1400 bp) gene and *Lox* (870 bp) were visualized by gel electrophoresis, *16S rRNA* and *Lox* genes were sequenced and analyzed with the NCBI Blast program, 16S rRNA and *Lox* genes has 98% and 99% homology compared with previously isolated strain (ATCC 29178) respectively. The sequence of the *16S rRNA* gene was uploaded to the NCBI Genbank database under accession number KY781829. The phylogenic tree analysis showed that *16S rRNA* gene of HNM-1 is evolutionary very close to previously isolated strains, it also showed the distribution of different isolates of *S. iniae* and other related species. Three clusters were observed in the phylogenetic tree, the first one corresponding to the root is *streptococcus lutetiensis*, followed by a defined group of *Streptococcus agalactiae* ATCC 13813 and *Streptococcus dysgalactiae* 172755 strains, at the top is a group comprising our isolate (HNM-1) and other *S. iniae* strains isolated from China, Japan, and Israel (Fig. 1).

*Antibiotic susceptibility*

The susceptibility of HNM-1 to 19 antimicrobial

agents is summarized in Table III. The bacteria is resistant to many important antibiotics that include Amoxicillin, ceftazidime, gentamycin, Penicillin G, piperacillin, polymyxin B, kanamycin and spectinomycin. This signifies an emergence of multidrug resistant strain which was not the case before.

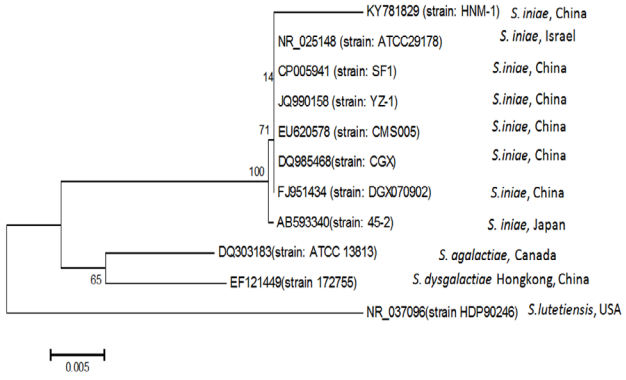


Fig. 1. Phylogenetic tree constructed based on *16S rRNA* gene sequence. The isolates were indicated by accession number, strain names, and country of isolation.

*Biochemical profiles of the isolate*

API 20 Strep kit was used for the determination of HNM-1 biochemical profile, and compared the result with previously isolated strains of *S. iniae* (Table II), reactions were consistently positive or negative in all the samples analyzed, Aesculin hydrolysis, pyrrolidonylaryl-amidase, alkaline phosphatase, Arginine dihydrolase reactions, and leucine-arylamidase reactions were positive but negative for Hippurate, acetoin, α-galactosidase, β-glucuronidase, and β-galactosidase. The bacterium was also able to produce acid from ribose, mannose, Trehalose, and glycogen, but no change in pH when grown in the presence of Arabinose, Sorbitol, lactose, Inulin, and Raffinose.

*Determination of* S. iniae *virulence genes*

6 significant virulence genes assayed in this study were all present in HNM-1 genomic DNA. Blast analysis of all the genes showed 99% similarities with previously isolated virulent strains of *S. iniae*.

*Scanning electron microscope of* S. iniae

It was well established that biofilm can protect the bacterial community from the innate immune system and antibiotics action (Chao *et al*., 2015). Scanning electron microscope revealed the structure of *S. iniae* arranged in chains as well as in clusters of communities (Fig. 2), indicating its ability to produce biofilm, which could contribute to its virulence and ability to overcome *A. sinensis* defense mechanisms (Nikolaev and Plakunov, 2007). Making it the first time to confirm the ability of *S. iniae* to produce biofilm.

**Table II. Comparison of *S. iniae* HNM-1 biochemical profile with that of other strains, the experiment was conducted using API 20 strep kit; the results were compared with that of previously isolated strains, indicated with alphabets A to E.**

| Test | HNM-1 | A | B | C | D | E |
|---|---|---|---|---|---|---|
| Acetoin production | - | - | - | + | - | ND |
| Hippurate | - | - | - | - | - | - |
| Aesculin hydrolysis | + | - | + | + | + | + |
| Pyrrolidonylaryl-amidase | + | + | + | + | + | ND |
| a-galactosidase | - | + | - | + | + | ND |
| b-glucuronidase | + | + | + | + | + | ND |
| b-galactosidase | - | + | - | - | + | ND |
| Alkaline phosphatase | + | + | + | + | + | ND |
| Leucinearylamidase | + | + | + | + | + | ND |
| Arginine dihydrolase | + | + | + | + | + | ND |
| Ribose fermentation | + | + | + | + | + | ND |
| Arabinose fermentation | - | - | - | - | - | - |
| Mannitol fermentation | + | + | + | + | + | + |
| Sorbitol fermentation | - | - | - | - | - | - |
| Lactose fermentation | - | - | - | - | - | - |
| Trehalose fermentation | + | - | - | + | + | + |
| Inulin fermentation | - | - | - | - | - | - |
| Raffinose fermentation | - | - | - | - | - | - |
| Starch fermentation | + | + | + | + | + | + |
| Glycogen fermentation | + | + | + | + | + | ND |
| β Haemolysis | β | β | β | β | β | β |

Notes: "+": positive reaction; "-" : Negative reaction; ND: not determined; HNM-1:current study; A: Jantrakajorn *et al*., 2014; B: El Aamri, *et al*, 2010; C: Rahmatullah *et al*., 2017; D: Suanyuk *et al*., 2010; and E: Pier and Madin, 1976.

*Reactive oxygen and whole fish blood survival assay*

One important mechanism by which phagocytic cells eliminate pathogens is through the release of reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Liu *et al*., 2005). Assay result revealed that HNM-1 has an average survival rate of 77% and 58% after 2 hours incubation at 2mM and 4mM concentrations of $H_2O_2$, respectively (Fig. 3A).

**Table III. Susceptibility of HNM-1to Antibiotics, 19 selected antibiotics were used in this study, the results were presented as susceptible or resistance according to Clinical and Laboratory Standards Institute (CLSI) guidelines.**

| Antibiotic Name | Paper content (µg/disc) | Zones of inhibition (mm) | Remarks | Standard inhibition zones diameter (mm) |
|---|---|---|---|---|
| Amoxicillin-clavulanic acid | 20 | 16 | R | S ≥ 18 |
| Cefazolin | 30 | 28 | S | S ≥ 23 |
| Cefotaxime | 30 | 30 | S | S ≥ 26 |
| Cefoxitin | 30 | 27 | S | S ≥18 |
| Ceftazidime | 30 | 15 | R | R ≤ 17 |
| Gentamicin | 10 | 13 | R | S ≥ 15 |
| Levofloxacin | 5 | 24 | S | S ≥ 17 |
| Lomefloxacin | 10 | 27 | S | S ≥ 22 |
| Norfloxacin | 10 | 26 | S | S ≥ 17 |
| Ofloxacin | 5 | 30 | S | S ≥ 16 |
| Oxacillin | 1 | 21 | S | S ≥ 20 |
| Penicillin G | 10 | 19 | R | S ≥ 24 |
| Piperacillin | 100 | 18 | R | S ≥ 21 |
| Polymyxin B | 300 | 10 | R | R ≤ 11 |
| Spectinomycin | 100 | 11 | R | R ≤ 14 |
| Ticarcillin - clavulanic acid | 75/10 | 41 | S | S ≥ 20 |
| Tobramycin | 10 | 24 | S | S ≥ 15 |
| Vancomycin | 30 | 20 | I | S ≥ 17 |
| Kanamycin | 30 | 12 | R | ≥18 |

S, susceptible; R, resistant.

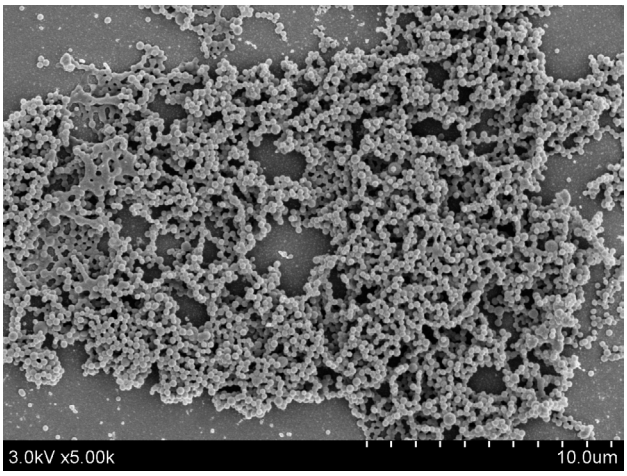


Fig. 2. Scanning Electron microscopic images of *S. iniae* HNM-1, Colonies were arranged in chains and in community, partially observed extracellular matrix-like structure of Biofilm can be observed.

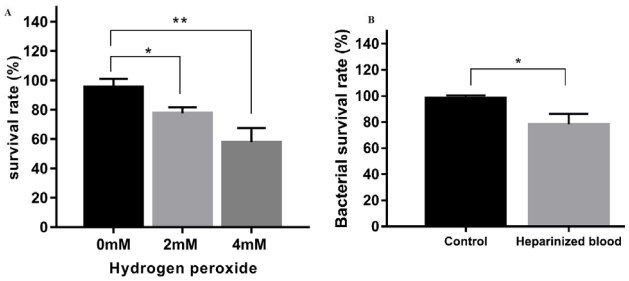


Fig. 3. Virulence of *S. iniae* HNM-1. (A) Percentage survival of *S. iniae* HNM-1 strain incubated in $H_2O_2$. The bacteria has a survival rate of 77%. (B) Percentage of CFUs after 2 hours of incubation at $35^0$C in heparinized fish blood.

To determine the percentage of *S. iniae* HNM-1 that can survive the innate immune response of *A. sinensis*, we examined its survival in whole fish blood. Up to 85% of the bacteria escaped macrophage and other innate immune responses killings and showed only a 15% reduced

survival rate compared with the control sample ($P < 0.05$) (Fig. 3B).

In vivo *infection assay*

Chinese Sturgeon infected with *S. iniae* HNM-1 strain manifested visible clinical signs, and symptoms, such as enlarged, pale liver, dark-colored kidney, and spleen, hemorrhage of internal organs, corneal opacity and accumulation of yellow fluid in the intestines, as well as erratic swimming and sluggishness, which were similar with the symptoms observed during field examination. *S. iniae* HNM-1 is highly lethal to Chinese Sturgeon at a concentration of 2.0 x $10^5$, 2.0 x $10^6$ and 2.0 x $10^7$ CFU $fish^{-1}$ which resulted in cumulative mortality of 40%, 80%, and 100% respectively (Fig. 4) and $LD_{50}$ of 1.6 x $10^6$ CFU, the control group maintained normal biological activities (with no deaths) during the experiment. *S. iniae* was re-isolated from the liver and spleen of the infected fish and confirmed its identity with 16S rRNA and *Lox* genes by PCR amplification to satisfied Koch's postulate.

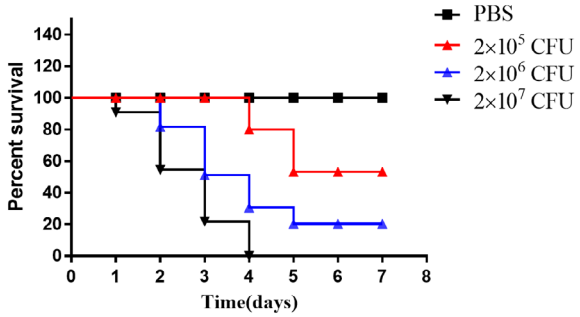


Fig. 4. Percent survival of *A. sinensis* challenged with different concentrations of *S. iniae* HNM-1.

*Histopathological examination*

Histopathological analysis of the infected *A. sinensis* revealed bacterial colonization and inflammatory cells infiltration of vital organs; the principal lesions in the heart includes loose myocardial, interstitial edema, thickened pericardium, and many inflammatory cells infiltration (black arrow in Fig. 5A). In the liver; hepatocytes were characterized with severe steatosis, cytoplasm filled with some round vacuoles of different sizes (black arrow in Fig. 5B); increased inflammatory cells in the sinusoids, and inflammatory cells infiltration in the portal area (yellow arrow in Fig. 5B). The brain structure is disorganized, the tissue is swollen and loose, accompanied by a large amount of inflammatory cells infiltration (black arrow in Fig. 5C). While in the spleen the boundaries of red and white pulps were unclear as well as diffuse spleen, lymphocyte necrosis (black arrow in Fig. 5D), accompanied by a small number of red blood cells infiltration and inflammatory cells enlargement (red arrow in Fig. 5D).

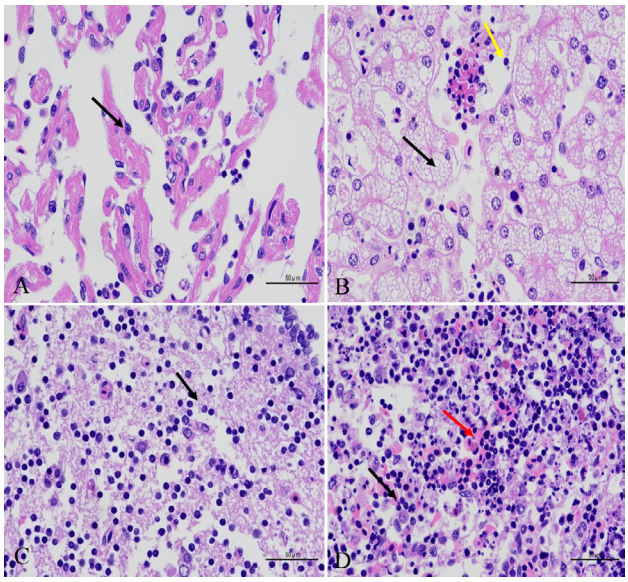


Fig. 5. Lesions observed in organs of Chinese sturgeon infected by *S. iniae*. The primary lesions in the heart include interstitial edema, enlargement of stroma as well as thickening of the pericardium (A). In the liver, hepatocytes were characterized by steatosis (B). Brain structure is disorganized with swollen and loose tissues (C). In the spleen, the boundaries of red and white pulps were unclear as well as splenic lymphocyte necrosis (D).

## DISCUSSION

Chinese sturgeon is an endangered fish species and essential delicacy in China. With the increasing susceptibility of sturgeon species to bacterial infections, it becomes mandatory to implement more measures and solutions to protect it from extinction. *Streptococcus iniae* remains one of the leading fish pathogens and responsible for causing morbidity and mortality of more than 30 fish species (Agnew and Barnes, 2007). In this study, we identified the etiological agent responsible for Chinese sturgeon (*Acipenser sinensis*) disease outbreak to be a strain of *S. iniae* ( HNM-1), which increased its host range and highlighted the alarming threat to the survival of the endangered fish species. characterization of *S. iniae* HNM-1 revealed: β- hemolytic, capsulated and gram-positive chained cocci, unable to grow at 10°C, 45°C and in 6.5% NaCl concentration, properties that differentiated it from *Enterococcus* and *Lactococcus* species (Schleifer and Kilpper-Bälz, 1987). Its ability to viably grow between 20°C to 35°C explained why its outbreaks are more prevalent

during summer. Likewise, its capability to grow optimally in a medium with salt concentrations from 0% to 3.0% gave it an ability to be lethal to both freshwater and marine fish species (Kia and Mehrabi, 2013). Biochemical profiles of *S. iniae* HNM-1 differs with most of the previously reported strains, for instance, it exhibits great variations in α-galactosidase, β-glucuronidase and trehalose reactions compared with the strains reported by (Rahmatullah *et al*., 2017; El Aamri *et al*., 2010; Jantrakajorn *et al*., 2014; Dodson *et al*., 1999), but comparable with the dolphin isolate which was the first isolated strain of *S. iniae* (Pier and Madin, 1976). Inconsistency in biochemical profiles is common among different strains of the bacteria, which can lead to frequent miss-identification of *S. iniae* using the biochemical method (Lau *et al*., 2003). We employed a molecular approach for its identification which successfully confirmed the responsible pathogen to be a strain of *S. iniae*. The phylogenic tree showed a clear geographical separation between groups, as well as genetic proximity, with Chinese and Israel isolates having more significant genetic similarities with *S. iniae* HNM-1 strain, which highlighted the probable relationship between them, as well as possible chain and source of transmission of the disease across China. Two possible ways of its spread could be due to the fact that not all the infected fish show apparent signs of the disease but can serve as a reservoir for future infection when used in fish feed as a source of protein (Zlotkin *et al*., 1998) or as a result of contamination of ponds by heavy rains or other forms of contaminants that may contains infectious bacteria (Creeper and Buller, 2006).

Antimicrobial susceptibility profile can help in effective chemotherapy to reduce mortality as well as avoid development of selective antibiotic resistance of, especially Zoonotic bacteria. *S. iniae* HNM-1 was found to be resistant to many important antibiotics that include Amoxicillin, gentamycin, Penicillin G and spectinomycin. This signifies an emergence of multidrug resistance strain unlike previously reported strains that were susceptible to most of the antibiotics (Rahmatullah *et al*., 2017; El Aamri *et al*., 2010; Kayansamruaj *et al*., 2017). The cumulative mortalities and $LD_{50}$ of HNM-1 strain in *A. sinensis* indicated the high vulnerability of *A. sinensis* to its infection.

The prominent symptoms manifested by both naturally and experimentally infected *A. sinensis* were of common *streptococcal* infection, the differences in clinical presentations between our results and previous studies could be attributed to age, genetic differences of fish species, virulence of the isolate and experimental conditions (Agnew and Barnes, 2007). We also determined its capability to produce biofilm which might contribute to its virulence. Scanning electron microscopy confirmed its ability to live in communities which can enable them to make biofilm as well as quorum sensing that are key to virulence and antibiotics resistance.

Histopathological examination revealed pathological lesions, a large number of bacteria in clusters, and massive infiltration of multi-systematic necrotizing inflammatory cells in the organs of the infected fish. These findings were consistent with the histological changes associated with *streptococcosis* (Ortega *et al*., 2018; Kayansamruaj *et al*., 2017; Deng *et al*., 2017).

Although the presence of virulence genes only may not necessarily correspond to expression of their virulent factors, but yet this result can serve as a fingerprint and subject for subsequent studies, such as allelic exchange mutagenesis, to fully understand their roles in the pathogenesis of the bacteria, which was limited in this study but part of our ongoing projects. In vitro reactive oxygen species killing assay for 2 h shows that 77% of the bacteria can survived in 2mM hydrogen peroxide, unlike non virulent strain of *S. iniae* which has 10 fold increased in susceptibility to hydrogen peroxide (Buchanan *et al*., 2008), in addition, the bacteria also survived whole blood killing (85%), which indicated resistance to oxidative killings of macrophages is a significant contributor for *S. iniae* survival.

## CONCLUSION

In this study we reported the first *S. iniae* infection of Chinese sturgeon (*A. sinensis* ), which increased the number of its host ranges and highlighted its increasing threat to the aquaculture industries. We also isolated and characterized the etiological agent. Furthermore *S. iniae* HNM-1 is resistant to most of the important antibiotics which is an alarming threat to the aquaculture industries and highlighted emergence of multidrug resisstant strain. The bacteria caused morbidity and mortality of Chinese sturgeon due to its ability to survived in blood, under oxidative stress, disseminated to various organs and caused fatal lesions. Furthermore, we determined *S. iniae* biofilm formation, effective chemotherapeutic treatments, as well as suggested preventive measures, such as an efficient vaccine, improvement of water quality, adequate quarantine, and low stock density as the best options for circumventing future disease outbreaks.

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*Statement of conflict of interest*

The authors declare no conflict of interest.

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