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Separation and identification of sphyraenus streptococcus pathogen

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ABSTRACT

A large number of sphyraenus bred in the Qiantang River reservoir of Zhejiang province died in 2013 and 2014 and the death rate reaches 40%. The diseased sphyraenus features exophthalmos and Intestinal hyperemia. To find the pathogen and avoid this disease, this paper studies the bacterial strain MSB0507 separated from the brain and liver of the sphyraenus. After animal regression test, the bacterial strain MSB0507 is identified as the pathogenic bacteria. After the bacterial strain morphology and bio-chemical identification, the streptococcus dysgalactiae is identified roughly as the disease-incurred bacteria. With 16S rDNA detection, PCR resultant sequencing and ribotide sequence comparison and retrieval, it is found that the homology of the streptococcus dysgalactiae with NCBI registration number AB537922 reaches 100%. The special prime is designed according to the surface protein MIG gene sequence of the streptococcus dysgalactiae and then the target section is grown smoothly. From the view of the molecular biology, this bacterium is identified as the streptococcus dysgalactiae. The medicine sensitivity test results indicate that this separated bacterial strain is sensitive to the antibiotic such as achromycin and soil enzyme. This paper proves that the streptococcus dysgalactiae incurs the disease of the sphyraenus and guides diagnosis and treatment of this disease.

KEYWORDS

Nitrite-oxidizing bacteria; 16S rDNA, polymerase chain reaction; Homology analysis.

INTRODUCTION

The sphyraenus belongs to Mugilidae family of the Mullet and is also called as redeye and meat stick. The wild sphyraenus mainly lives in the confluence of the saline water and fresh water at the coastal and river ports in China. This type is highly adaptive to salt degree and temperature and is the omnivorous fish with the plant bait as the food. This fish is one of the main breeding fish in coastal areas such as Zhejiang and Jiangsu province and is mainly bred together with the grass carp, crucian and South America whiteshrimp[1]. In recent years, partial farmers start to mainly breed sphyraenus. With increase of the breeding scale and density, the old sphyraenus with stronger disease resistance also die in a large scale. a large number of sphyraenus bred in the Qiantang River reservoir of Zhejiang province died in 2013 and 2014 and the death rate reaches 40%. This test aims to separate the disease-incurred bacterial strain from the diseased sphyraenus and the physiological and bio-chemical feature analysis, 16S rDNA gene sequence research and drug sensitivity test are performed for this bacterial strain.

MATERIALS AND METHODS

Bacterial strain separation

The diseased sphyraenus from Qiangtang River reservoir of Zhejiang province features exophthalmos, small intestinal hyperemia and small ascites inside the abdominal cavity. The liver, brain and kidney tissues are taken under the sterile conditions and are placed on the brain and heart infusion plate (BHIA) for streak culture under 300C. after 48h, the single colony is taken for pure cultivation.

Observation of pathogen shape

Take the purified single colony, cultivate under 30℃ for 18h, smear, fix and dye, and observe it under the microscope.

Toxicity test of separated bacterial to sphyraenus

Pick up the colony, vibrate and cultivate in the brain and heart infusion cultivation liquid for 24 h, make the bacterial suspension liquid with the sterile normal saline, adjust the bacterial liquid concentration as 10^7, 10^6, 10^5 and 10^4 and inject the bacterial liquid with different concentrations into the muscle of healthy sphyraenus (root of dorsal fin). 10 sphyraenuses are used in each test and are 10-15cm long. The sphyraenus injected with the normal saline is used as the contrast group. The sphyraenus are bred in the water cluster cylinder. The cylinder is 1m long, 1m wide and 1m deep. The water temperature is 25℃. The disease conditions are observed. The bacterial liquid is injected into the back muscle of the healthy sphyraenus. The sphyraenuses in the contrast group are injected with the normal saline. Each sphyraenus is injected with 0.3ml bacterial liquid and the concentrations are 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4cfu/mL and 1.0×10^3cfu/mL and the blank contrast group is set.

Identification of bio-chemical features

The France BioMerieux ATB automated bacteria identification instrument, Rapid ID 32 Strep quick streptococcus and related bacteria identification test box are used for bio-chemical identification of the purified bacterial strain.

PCR identification of separated strain

Primer design: the significant 16S rDNA in the bacteria taxology is used as the target gene. 1360bp is used as the target fragment. The primer sequence is shown as follows[2]:

Seq forward：5'-GAGCGGATAACAATTTCACACAGG-3'

Seq reverse：5'-CGCCAGGGTTTTCCCAGTCACGAC-3'

The special primer is designed according to the surface protein MGI gene sequence of the Streptococcus dysgalactiae[3]. The target fragment is 370bp. The primer sequence is shown as follows:

Seq forward: 5'-AACTGGAGGAAGGTGGGGAT-3'

Seq reverse: 5'-AGGAGGTGATCCAACCGCA-3'

The primer is made by Shanghai Yingjun Biology Technology Co., Ltd.

PCR reaction: Extract DNA by using the boiling method. Conditions of PCR reaction: 50μL reaction system, add 5.0μL 10×PCR-Buffer, 4.0μL 25mmol/L $MgCl_2$, 4.0μL 2.5mmol/L dNTP, 0.3μL 5U/μL Taq polymerase, 1.0μL 10pmol/L upstream and 1.0μL 10pmol/L downstream primer, add 33.7μL purified water and 1μL template, pre-denature it under 94℃ for 3min, expand PCR under 94℃ for 1min, under 54℃ for 1min and under 72℃ for 1min, perform for 30 cycles, and extend under 72℃ for 7min. Detect PRC resultant with 1.5% agarose gel electrophoresis, and dye with ethidium bromide[4]. The PCR resultant sequencing is completed by Shanghai Yingjun Biology Technology Co., Ltd.. Later the nucleotide sequence is compared and retrieved (Blast).

Drug sensitivity test

The drug-sensitive slip agar flat plate diffusion method[5]. The drug-sensitive slip is purchased from Hangzhou Tianhe Microbial Reagent Co., Ltd.. Adjust the bacterial liquid concentration to 1.5×10^8cfu/mL according to the operation manual, take 20uL bacterial liquid equably on the blood flat plate, and stick the drug-sensitive slip on the flat plate surface. Notice that each slip should keep enough distance and observe results after 24h.

RESULTS

Observe with thallus shape microscope

Separate smooth and round small colony from the brain and heart infusion liquid flat plate, pick up pure cultivated single colony MSB0507 for smearing and dying, and observe chain rank of the spherical bacteria of different lengths with the microscope. Blood plate: to cultivate under 37℃ for 24 hours, β hematolysis small colony and gram's staining positive (G+) streptococcus with short chain will grow and the bacitracin is negative.

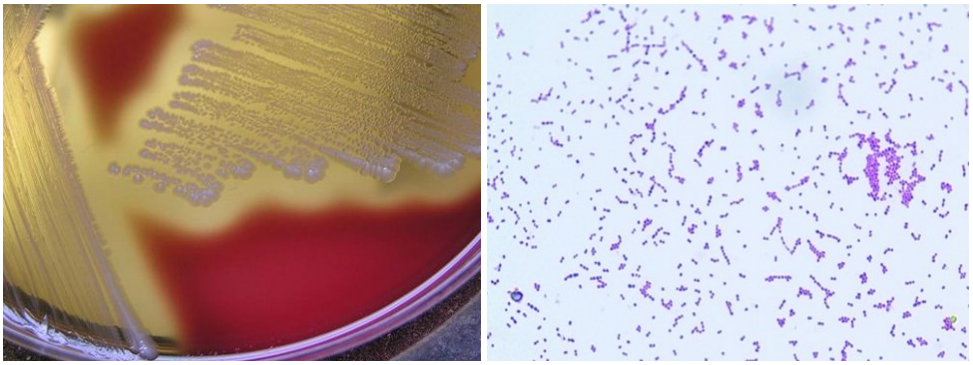

Figure 1 : Hematolysis photo and microscope shape of separation bacteria MSB

Physiological and biochemical identification

Marcel Merieux is used to identify bacterial strain MSB. Its bio-chemical spectrum is shown as follows:
ADH + BGLU- BGAR- BGUR+ Agal- PAL+ RIB+ MAN- SOR- LAC- TRE+ RAF- VP- APPA+ BGAL- PYRA- BNAG- GTA- HIP- GLYG- PUL- MAL+ MEL- MLZ- SAC+ LARA- DARL- MBDG- TAG- BMAN- CDEX- URE-
To compare the bio-chemical spectrum, Streptococcus dysgalactiae is identified (*S. dysgalactiae*).

Toxicity injection test

TABLE 1 : MSB0507 bacterial strain injection test for sphyraenus

Concentration of bacterial liquid	Dosage of injected bacteria	Number of test sphyraenus (piece)	Number of dead sphyraenus (piece)
1.0×10^7	0.3	10	10
1.0×10^6	0.3	10	8
1.0×10^5	0.3	10	5
1.0×10^4	0.3	10	3
1.0×10^3	0.3	10	0
normal saline	0.3	10	0
Blank contrast	0.3	10	0

From the bacteria injection, this bacterial strain is very toxic to the healthy sphyraenus and the dosage LD_{50} for leading to death of half sphyraenus is 2.38×10^4 cfu/ml. Take the brain, liver and kidney tissue of the sphyraenuses which are dead on third day after bacteria injection, separate the bacteria and get the bacterial strain which growth feature is same as it of MSB0507. The colony on the separation flat plate is plentiful and pure and has no other bacteria, so it meets the Koch postulates law.

PCR identification of separated bacterial strain

16S rDNA gene fragment of the bacterial strain in PCR is 1360bp. To return this fragment, measure sequence, and analyze homology of this sequence, it is found that the gene is 100% homology with the Streptococcus dysgalactiae bacteria strain registered in America Gene Bank (registration number is AB537922 and EU075033).

The special primer has a clear and bright belt at 370bp (figure 2). The belt 1 is for the separated bacterial strain. The belt 2 is for blank contrast, the belt is for suppurative streptococcus ATCC19615 and the belt is for golden staphylococcus ATCC29213.

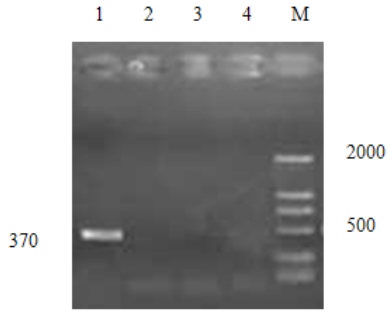

Figure 2 : PCR identification of special MIG gene of separate strain

The homology analysis of the sequence comparison analysis of 16S rDNA sequence and the special primer growth results of the surface protein MIG gene sequence of the streptococcus dysgalactiae further prove that this separation strain is the streptococcus dysgalactiae.

Drug sensitivity test

The results indicate that roxithromycin, norfloxacin, doxycycline, penicillin G, ceftriaxone and lincomycin have any suppression effect on two strains, but the separated strain is sensitive to achromycin, cefalexin IV, oxytetracycline, enoxacin, ciprofloxacin and neomycin. The inhibition zone data of several antibacterials to the strain are shown as the TABLE 2.

TABLE 2 : Inhibition zone diameter of several antibacterials to the strain MSB0507

Antibiotics Diameter of inhibition (mm) Result	Antibiotics Diameter of inhibition (mm) Result
Tobramycin 18 M	oxytetracycline 20 S
Roxithromycin 0 R	Enoxacin 22 S
Gentamicin 12 R	Ciprofloxacin 28 S
Enrofloxacin 14 M	tetracycline 22 S
Norfloxacin 0 R	Kanamycin 16 M
Cefalexi 21 S	Rocephin 0 R
Doxycycline 0 R	Neomycin 20 S
Penicillin 0 R	Streptomycin 12 R
Furazolidone 10 R	Lincomycin 0 R

Remark: S indicates sensitive, R indicates resistant, and M indicates moderately sensitive.

DISCUSSION

With the pathogenic isolation and identification and regression infection trial, the strain MSB0507 is the nosophyte which leads to exophthalmos, small intestinal bleeding and small ascites of sphyraenus and incurs a large scale of dead sphyraenus. This experiment identifies the species of the streptococcus pathogen via the biochemical and molecular biology and confirms the streptococcus dysgalactiae as the pathogen. The streptococcus dysgalactiae is diversified, is distributed in the nature extensively, and is one of main pathogens of the streptococcus. It can generate multiple toxins and toxic enzymes. Now the streptococcus becomes one of the main pathogenic bacteria for the fish and will endanger multiple fresh freshwater fish and saltwater fish such as salmon trout, snapper sea bream, flatfish, ayu and river salmon. It will lead to higher death rate. It is reported that the Streptococcus iniae[6] is separated from the red drum, Streptococcus faecalis[7] is separated from rainbow trout, Streptococcus shiloi and Streptococcus difficile[8,9] are separated from tilapia mossambica and Streptococcus agalaciate[10]is separated from rana catesbiana. The streptococcus also frequently leads the disease of the human being, e.g. suppurative streptococcus generating the dick toxin will also incur scarlatina and dolphin streptococcus can easily incur a disease to the human being. The streptococcus agalactiae will lead to sepsis, meningitis and pneumonia of infants[11], so the

fish diseases incurred by the streptococcus not only endangers the fishing industry, but also threatens the food security and human being health, so it is highlighted to diagnose, detect and treat the streptococcal pathogens.

The sphyraenus bred in Hangzhou Bay of Zhejiang province suffers from the streptococcus dysgalactiae. The sphyraenus features slow movement, anorexia and unbalance in the initial period. Partial sphyraenus features prominent eyes in the late period. A small number of ascites is found in the abdominal cavity via autopsy, but the diseases incurred by different streptococcus have different symptoms for different fish. E.g. The diseased rainbow trout features the blood cake and bleeding around the anus. The diseased hamachi features bleeding inside operculum and enteritis. The diseased tilapia mossambica features prominent eyes, unbalance, and ascites. Sometimes, if the brain is injected, the above symptoms will not occur and the diseased fish will acutely die. The streptococcus can only be separated from the organs [12]. This experiment designs the special primer of the surface protein MIG gene sequence of the streptococcus dysgalactiae, which can improve detection speed and accuracy of the streptococcus disease, can take drug in time for this bacteria, and indirectly reduce the loss incurred by the streptococcus disease to the farmers.

The results of the drug sensitivity experiment indicate that the separated strain is sensitive to tetracycline, cefalexi, oxytetracycline, enoxacin, ciprofloxacin and neomycin. The sphyraenus is one important economical aquiculture breed, so the drug should be correctly and reasonably taken for the diseased sphyraenus to secure the aquiculture foods. To further establish a reasonable breeding mode and study the infection mechanism and Infectious factors of the nosophyte can reduce occurrence of this pathogen, effectively prevent against and control the streptococcicosis, and ensure healthy and sustainable development of the sphyraenus breeding industry in China.

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