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Full Length Research Paper

Examination of red-leg disease and its pathogen, *Rana temporaria chensinensis*

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Examination of pathogenic bacteria, isolated from *Rana temporaria chensinensis,* was conducted including morphological characteristics, physiological and biochemical characteristics, as well as the detection of the 16S rRNA gene sequence of the isolates. Phylogenetic tree based on the two sequences (HQ070610A-1, HQ070610B-1) and homologous sequences from GenBank was constructed by neighbor-joining method. Meanwhile, the pathogenic effect of the representative strains to healthy *R. temporaria chensinensis* was determined, the susceptibility to antimicrobial agents was conducted with agar diffusion method (Kirby-Bauer, K-B) and the haemolysin A gene was detected by PCR amplification. The isolated strains were identified as *Aeromonas hydrophila*. The length of 16S rRNA gene of strain HQ070610A-1(GenBank accession No.GQ470995) was 1444bp, and the length of 16S rRNA gene of strain HQ070610B-1(GenBank accession No.GQ470996) was 1445bp. The high pathogenicity of the representative isolated strain to healthy *Rana c.* could be confirmed by challenge test. All strains were sensitive or highly sensitive to 28 agents including cefotaxime, cefotaxime etc., and they were slightly sensitive to cefazolin, resistant to 8 agents including penicillin G, vancomycin, etc. The eleven isolated strains all carried haemolysin A gene.

Key words: *Rana temporaria chensinensis*, red-leg disease, *Aeromonas hydrophila.*

INTRODUCTION

Rana temporaria chensinensis is commonly called frog, which is the Chinese subspecies of *Rana temporaria*. It belongs to Ammphibia, Anura, Ranidae and Frog. The fallopian tubes of female (Rana oil) are a rare medicinal herb, and the meat is delicious in food because of the tender and good taste. Therefore, artificial breeding of diverse modes was recently carried out in China. Because the living surroundings between nature and feeding were quite different, for example the pen, railings, ventilation etc., various diseases occur frequently. Especially the communicable diseases caused by some pathogenic microorganisms which often show higher morbidity and mortality, which had constituted serious threat to the healthy cultivation of Rana (Yan and Zhang 2005; Zhou et al., 2007; Han et al., 2006; Liu et al., 2000). broke out with diseases that caused death in a *R.*

In June 2007, the cultured breeding frogs frequently *temporaria* farm in Hebei, China. Diseased frogs showed symptoms including psyche weakness, loss of appetite and stopped feeding. Examination of diseased and dead frogs had indicated that the disease was caused by *Aeromonas hydrophila.* This paper is to report the incidence of *R. temporaria*, main biological characters of corresponding pathogenic bacteria, as well as to comprehend condition of haemolysin (hlyA), which provide references for the effective test, prevention of red-leg disease, and the further study of *A. hydrophila.*

MATERIALS AND METHODS

Examination of case

Examination of pathological changes, smears prepared, Gram stained and examined microscopically were carried out for the diseased and dead frogs. Both isolation and identification of pathogen based on above observations were performed.

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Isolation and pure cultivation of the strains

Bacteriological samples from fresh dead frogs (number: 1, 2, 3 and 4) were taken aseptically from liver and ascitic fluid (from the sample NO. 4). The samples were streaked for isolation onto nutrient agar plates, and incubated at 28°C for 24 h. After incubation, morphologically similar and dominant bacterial colonies were selected and sub cultured onto nutrient agar to obtain pure cultures for identification.

Identification of the isolates

Examination of morphologic characteristics of the isolates

Pure isolated colonies were transferred onto nutrient agar and incubated at 28°C for 18 h. After incubation, the smear preparations of the pure isolated colonies were made, and their morphology examined by microscope after Gram staining.

Examination of bacterial colony characteristics

Pure isolated colonies were streaked onto nutrient agar plates, blood agar plates, and incubated at 28°C for 24 and 48 h, respectively. Then the growth information and bacterial colony characteristics were examined.

Determination of main physiological and biochemical characteristics

The physiological and biochemical traits of the pure cultures were carried out using the standard tests including oxidase, catalase, sugar (alcohol) metabolism, and organic acids salt utilization etc., according to Manual of Determinative Bacteriology to Usual Bacteria (Dong and Cai, 2001).

16S rRNA gene sequencing and phylogenetic

Representative strains HQ070610A-1 and HQ070610B-1 were inoculated into nutrient broth, and incubated at 28°C for 16 h. Then, DNA of the strains were extracted according to MiniBest Bacterial Genomic DNA extraction kit (TaKaRa Biotechnology Co., Ltd, Dalian, P. R. China) and acted as the template of PCR.

The 16S rRNA gene of the isolate was amplified by PCR using the bacterial universal primers (27F, 5°C-AGA GTT TGA TCM TGG CTC AG-3°C;1492R, 5°C-TAC GGM TAC CTT GTT ACG ACT T-3°C) (Martin and Collen 1998), 0.4 µl dNTP, 1 µl template DNA. PCR conditions as follows: initial denaturation at 95°C (6 min) followed by 30 cycles of denaturation at 94°C (1 min), annealing at 55°C (45 s) and extension at 72°C (1 min). PCR products were purified by DNA purification system (Wizard PCR Preps, Promega) and sequenced using 3730 sequencing machine by BoYa Biotechnology Corporation, Shanghai, China.

The 16S rRNA sequence was aligned and compared with available sequences in the NCBI GenBank using BLAST. Phylogenetic trees were constructed by initially aligning the sequences using CLUSTAL X version 1.8 with 1000 bootstraps by neighbor-joining method.

Determination of isolations classified situation

All pure cultures were identified based on their morphology, colony, physiological and biochemical characteristics, following the criteria described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and Bergey's Manual of Systematic Bacteriology (George, 2005). In addition, the results of 16S rRNA gene sequence and phylogenetic tree were applied as supplementary.

Experimental infection

The suspension of bacteria was made by inoculating the representative strains (HQ070610A-1 and HQ070610B-1) into nutrient broth, and incubated at 2°C for 18 h. For HQ070610A-1 strain, 4 healthy frogs were infected by injecting hind limb with 0.1 ml of 3 × 10^7CFUml^{-1} nutrient broth per frog, and for HQ070610B-1 strain was ditto. In addition, 4 healthy frogs were injected with 0.1 ml nutrient broth as controls. All the frogs were separately maintained in tanks. The disease and death of all frogs were observed after infection. The pathogenicity of the isolates was determined by duplicating the same infections as natural outbreaks and reinsulated the bacteria which were used in experimental infection.

Antimicrobial susceptibility of isolated strains

Antimicrobial susceptibility was conducted with agar diffusion method (K-B). Sensitivity and resistance to the antimicrobial agents were determined after 24 h (at 28°C) based on diameters of inhibition zones (Ye and Wang, 1997).

Haemolysin A (hlyA) genes detection by PCR amplification

The hlyA genes Primer was synthesized by Biotechnology Limited Company, Shanghai. Primer subsequence: F, 5°C-GCC GAG CGC CCA GAA GGT GAG TT-3′; R,5′- GGT ATG TTC AAA GCC CTG GAA GGA G-3′ (Hu et al., 2006). PCR amplification of the hly A gene was performed in a total volume of 25 µl containing 12.5 µl 2 × PCRmix，0.5 µl F primer，0.5 µl R primer, 2 µl target DNA, and 9.5 µl sterile water. PCR conditions were performed as follows: initial denaturation at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 62°C (50 s), extension at 72°C (1.5 min), and extension at 72°C (10 min) at last. Then, haemolysin A genes was detected according to the result of 1% Agarose Gel Electrophoresis.

RESULTS

Incidence and pathological change characteristic

Thirteen frogs (8 diseased frogs and 5 fresh dead frogs) were detected and found hyperemia, hemorrhage in the legs and gastrointestinal hemorrhage. Three frogs were found with hydroabdomen, intramuscular bleeding in the legs, which showed the obvious characteristics of red-leg disease.

Bacteria of lesion tissues

There were a large number of bacteria in the liver and hydro peritoneum of 4 diseased or dead frogs, which were Gram-negative, occurring singly or doubly individually, no spore, round-ends, rod shape with the

Table 1. Distribution of isolates.

Number	Liver		Subcutaneous muscle tissues		Ascites fluid	
	Strain A	Strain B	Strains A	Strain B	Strain A	Strain B
1	+	+	+	+	·	·
2	−	+	−	+	·	·
3	−	+	−	+	·	·
4	−	+	−	·	−	+

Represents no the item.

Table 2. Origine and numbers of pure culture strains.

Number	Liver		Subcutaneous muscle tissues		Ascites fluid	
	Strain A	Strain B	Strain A	Strain B	Strain A	Strain B
1	HQ070610A-1	HQ070610B-1	HQ070610A-2	HQ070610B-2	·	·
2	·	HQ070610B-3	·	HQ070610B-4	·	·
3	·	HQ070610B-5	·	HQ070610B-6	·	·
4	·	HQ070610B-7	·	HQ070610B-8	·	HQ070610B-9

size of 0.6 ~ 0.8 × 1.2 ~ 2.0 μm.

Isolation and pure cultivation of the strains

Pure cultures were obtained from liver, subcutaneous muscle tissues and ascites fluid of 4 diseased and dead frogs. The isolations were divided into strain A and strain B. Strain A colonies were smooth, circular (1.2 mm diameter after 40 h at 28°C), slightly convex, entire, slightly opaque, greyish-white, and well grown. While diameter of Strain B colonies were 1.5 mm and opaque. Distribution of two strains in different separated tissues is shown in Table 1.

Eleven isolated colonies were inoculated onto nutrient agar slant and incubated at 28°C for 24 h and 48 h, including 2 strains A and 9 strains B. The origin and number are shown in Table 2.

Identification of the isolates

Morphologic characteristics

Eleven pure cultures were separately inoculated onto nutrient agar slants and incubated at 28°C for 20 h. After incubation, the smear preparations of the pure cultures were made, their morphology was examined by microscope after Gram staining. The result showed that the morphological characteristics of 2 strains in strain A (HQ070610A-1 and HQ070610A-2) were found to be consistent, Gram-negative, occurring singly or doubly individual, no spore, round-ends, rod shape with the size of 0.4 ~ 0.7 × 1.0 ~ 1.5 μm; Nine strains in strain B (form HQ070610B-1to HQ070610B-9) were found to be consistent, the morphological characteristics were similar to strain A, rod shape with the size of 0.5 ~ 0.7 × 1.0 ~ 2.0 μm.

Cultural characteristics

Eleven pure cultures were inoculated in different medium plates respectively, examined after 24 h and 48 h at 28°C. The results showed that the colony characteristics and growth of HQ070610A-1and HQ070610A-2 on nutrient agar were corresponding with those from natural cases, which were basically identical on blood nutrient agar. Colonies were narrow with β haemolysis, approximately 1.3 and 2.0 mm in diameter after 24 and 48 h, respectively. On SS agar medium, colonies were circular, smooth, entire, slightly convex, colorless, opaque, approximately 1.2 mm in diameter after 24 h with good growth.

The colony characteristics and growth of HQ070610B-1 to HQ070610B-9 on nutrient agar were corresponding with those from natural cases, which were basically identical to blood nutrient agar. Colonies showed β haemolysis, approximately 1.5 mm and 2.0 mm in diameter after 24 and 48 h. On SS agar medium, colonies were circular, smooth, entire, colorless, opaque, approximately 2.0 mm in diameter after 24 h with good growth.

Identification and characterization of physiological and biochemical

Physiological and biochemical traits of 2 strains A (HQ070610A-1 and HQ070610A-2) and 9 strains B

Table 3. The item and result with discrepant response.

Item	Strain A	Strain B	Item	Strain A	Strain B
citrate	-	+	malonate	-	+
MR test	+	-	salicin	+	-
V-P	_	+	cellobiose	+	-
Esculin	-	+	α-methyl-D-glucoside	-	+

Notes: +, positive; -, negative.

(HQ070610B-1 to HQ070610B-9) were identical, respectively. All positive reactions included growth at 3°C, Oxidase, catalase, O-F test (F), motility, Gelatin hydrolysis, glucose acid production and gas production, lecithinase, indole, phenylalanine deaminase, proteinase, diastase, lipoidase, dextrin, fructose, nitrate reduction, glycerol, trehalose and DNAase. All negative reaction items included H_2S, sorbose, melibiose, sucrose, rhamnose, inositol, arabinose, mannitol, urease, maltose, xylose, Adonitol, melampyrin, mannose, galactose, inulin, raffinose and lactose. All conflicting reaction results are shown in Table 3.

16S rRNA gene sequence and phylogenetic

16S rRNA gene sequence and phylogenesis of representative strains HQ070610A-1 and HQ070610B-1 were analyzed. The results showed that the length of 16S rRNA gene of strain HQ070610A-1(GenBank accession No.GQ470995) was 1444bp, while the length of 16S rRNA gene of strain HQ070610B-1(GenBank accession No.GQ470996) was 1445bp. The 16S rDNA sequence of representative strains HQ070610A-1 and HQ070610B-1 were compared with available sequences in the NCBI GenBank using BLAST. The result showed that a high similarity (99%) with the 16S rRNA partial sequences of strains belong to Aeromonas. Phylogenic analysis of 16S rRNA gene sequence of some strains were carried out, and the phylogenetic tree representing genetic relatedness among the isolates and twenty-two 16S rRNA gene sequence from GenBank database are presented in Figure 1.

The isolates were identified as *A. hydrophila* based on their morphological, physiological and biochemical characteristics, as well as the 16S rRNA gene sequencing and phylogenetic.

Experimental infections

The infection experiment of strains HQ070610A-1 and HQ070610B-1 were carried out according to the previous descriptions. The result showed that two of four frogs infected by strains HQ070610A-1 died in 24 h, and then one died in 48 h. All four frogs infected by strains HQ070610B-1 died in 24 h. All diseased and dead frogs were detected with obvious hyperemia or hemorrhage in the legs, a little of ascites and gastrointestinal hemorrhage or abdominal tympanites, consistent with the natural infected cases of red-leg disease. The smears of liver from above died frogs were examined by Gram-stain. The results showed that a lot of Gram-negative rods occurred, which consistent with infected bacteria. In addition, the representative strains were reisolated respectively from the liver of infected mortalities, which were inoculated on nutrient agar and blood nutrient agar (contain 7% rabbit blood) and incubated at 28°C. Strains isolated from the infected frogs were obtained in pure cultures, and several tests were performed as described earlier. The results were consistent with the originally infected strains. Four frogs in the control group and one frog which were infected by strain HQ070610A-1 maintained alive throughout experimental period (10d).

Antimicrobial susceptibility

The sensitivity of representative strains HQ070610A-1 and HQ070610B-1 to 37 antimicrobial agents was determined. The results showed that there was no obvious variance between them. Two strains were susceptible or hypersensitive to cefotaxime, ceftriaxone, ceftazidime, cefoperazone, cefepime, aztreonam, erythromycin, azithromycin, streptomycin, kanamycin, gentamycin, tobramycin, amikacin sulphate, neomycin, spectinomycin, norfloxacin, ofloxacin, ciprofloxacin, tetracycline, doxycyline, chloramphenicol, polymyxinB, rifampicin, sulfamethoxazole, trimethoprim, nitrofurantoin, furazolidone and enrofloxacinum (diameters of inhibition zone were in the range from 16 to 40 mm); moderately susceptible to cephazolin (diameter of inhibition zone was 14 mm) and resistant to cefradine, vancomycin, novobiocin, penicillin G, oxacillin, ampicillin, clindamycin and bacitracin.

Detection of haemolysin A (hlyA) genes

PCR amplification was carried out for 11 strains. The result showed that all the strains had specific bands, which was approximately 309 bp and agreed to the

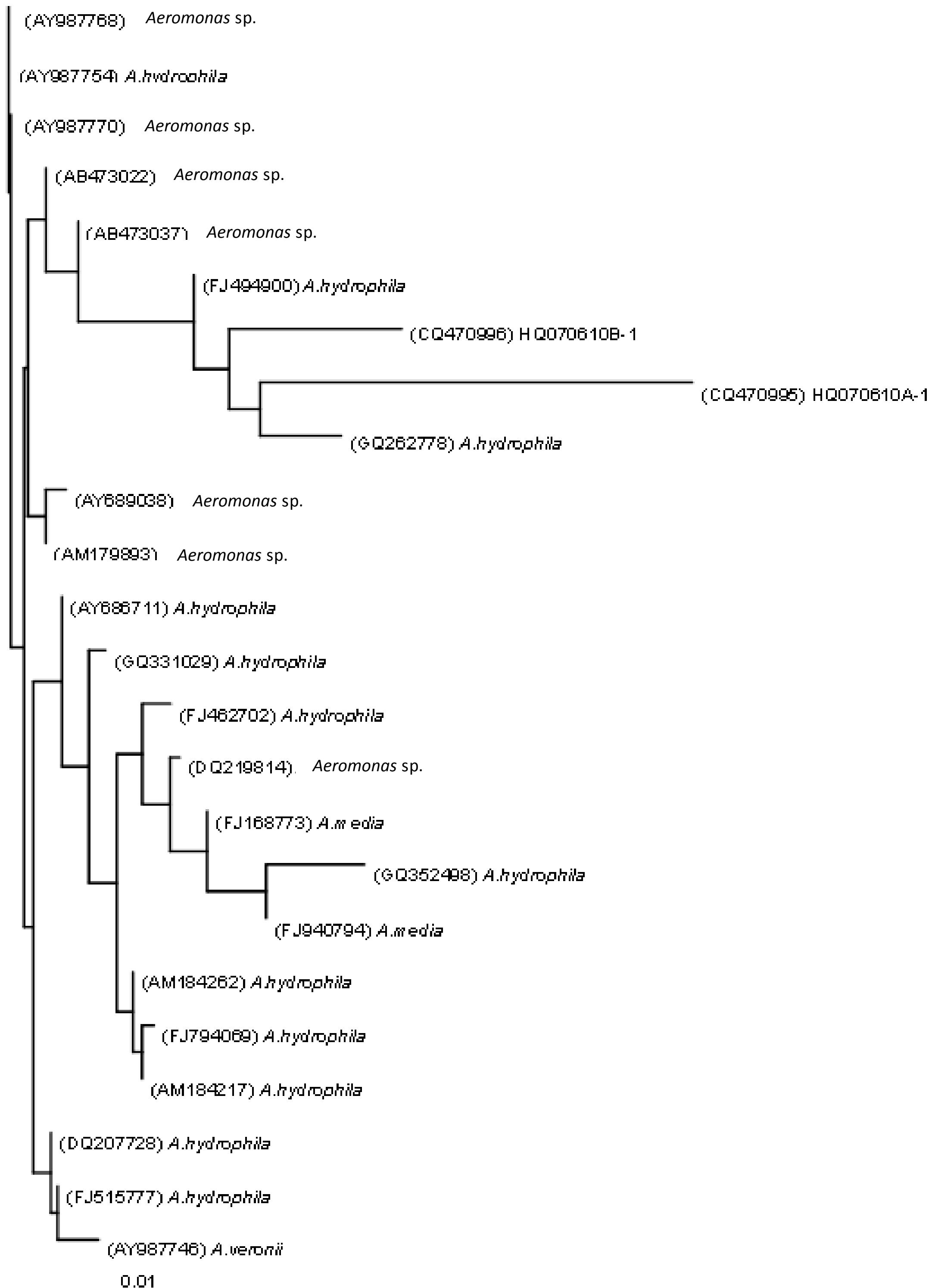

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences (AY987768~ AY987746 were database accession numbers in NCBI).

expected product (Figure 2). The bands of strains HQ070610B-2 to HQ070610B-9 were especially bright, and the others were slightly dark.

DISCUSSION

A. hydrophila is the cause of red-leg disease, and it was

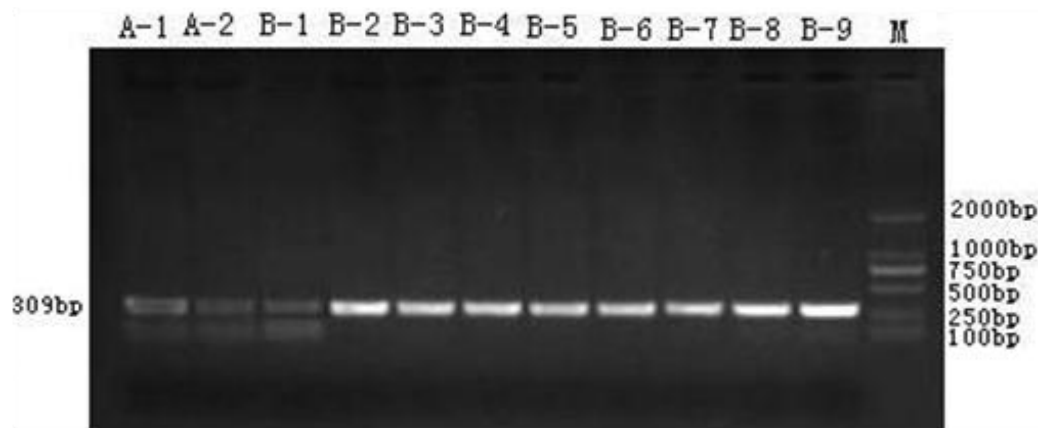

Figure 2. PCR amplification result of haemolysin A genes.

first isolated from infected frogs by Sanarelli in 1891. At present, the research results show that it is recognized as the pathogen of fishes and other aquatic animals and widespread in the world, and it has brought the most serious consequence for aquaculture (Xu et al., 1993; Dong et al., 2002; Cheng et al., 1999; Zhang et al., 1999; Austin and Austin 1999).

The infected frogs showed the relatively typical symptoms of red-leg disease in the examined case. All the isolates were identified as *A. hydrophila* by pathogen testing. The main clinical symptoms infected by *A. hydrophila* in frogs were hyperemia or hemorrhage in the legs, especially in the hind limb. However, based on the characteristic of bacteria distributed all over the body, it should belong to a type of septicemia. All the strains contain haemolysin A (hly A) gene, which should be the main reason of leading to septicemia. On the other hand, strain A was different from strain B on some physiological and biochemical characteristics. Although all the isolates were identified as *A. hydrophila*, we might suggest that strain B was the primary pathogen according to the isolated rate from natural cases, and the virulence was obviously higher than strain A in infection experiment.

Detected *A. hydrophila* were divided into two types. The differences of their biochemical indicator were showed as citrate, V-P, malonate, MR test, Esculin, α-methyl-D-glucoside, salicin, cellobiose etc. However, they were consistent with each other on the other main biochemical indicator of *A. hydrophila*. The result of phylogenetic tree showed that they formed a branch. Obviously, the species identification of *Aeromonas* should emphasize comprehensive judgment, and this also corresponded with the demarcation tendency of species in *Aeromonas* at present.

The antimicrobial susceptibility of two types of strains to experimental antimicrobial agents did not show obvious differences. The result may provide a reference to the resistance spectrum and the rule of drug resistance of *A. hydrophila*, and a guide to the selection of drugs to prevent the infections caused by these bacteria.

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REFERENCES

Austin B, Austin DA (1999). Bacterial Fish Pathogens: Disease of Farmed and Wild Fish [M]. Third (Revised) Edition. Praxis Publishing Ltd, Chichester, UK, pp. 19, 64-65.

Cheng TY, Tu XD, Hu BX (1999). On the Pathogenic Bacteria of the Ascitesosis of Trionyx Sinensis [J].Journal of Xinyang Teachers College. Nat. Sci. Edition, 12 (4): 451-455.

Dong CF, Lin TL, Chen R, Chen Q (2002). Studies on Pathogencitices and Biochemical Characteristics of Pathogens which Caused *Anguilla japonica* Bacterial Septicaemia and Ascites [J]. Fisheries Sci., 21(1):5-8.

Dong XZ, Cai MY (2001). Manual of Determinative Bacteriology to Usual Bacteria [M]. Beijing: Science Press, pp. 106-119,353-398.

George MG (2005). Bergey's Manual of Systematic Bacteriology [M]. Second Edition. Volume Two. Part B. Springer, New York, pp. 556-572.

Han XL, Yan DR, Liang K (2006). Isolation and Characterization of Pathogen which Caused Cultured Wood Frog Dead and Drug Sensitive Test [J]. Tech. Advisor Animal Husbandry, 8:38-39.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994). Bergey's Manual of Determinative Bacteriology [M]. Ninth Edition. Baltimore, Williams and Wilkins, pp. 73,129,190-191,253.

Hu J, Li AH, Hu CY, Zhu D L, Cai TZ, Wang JG, Si YD (2006). Effects of Temperature and pH on Expression of Virulence Factors from *Aeromonas Hydrophila*. J. Nanjing Univ. Sci. Technol., 30(3): 375-380.

Liu T, Qian AD, Xu DK, Liu QH (2000). Isolation and Characterization of Pathogen which Caused Wood Frog Red-leg Disease [J]. Chinese J. Veter. Drug, 34(4):39-41.

Martin FP, Collen MC (1998). Bias in Template to Product Ratios in Multitemplate PCR, J. Appl. Environ. Microbiol., 64 (10): 3724-3730.

Xu BH, Yin Z, Wu YS, Cai TZ (1993). Studies on the Taxonomy of Pathogenic Bacteria of the Bacterial Hemorrhagic Septicemia in Cultured Fishes in Freshwater [J]. Acta Hydrobiologica Sinica, 17(3):259-266.

Yan DR, Zhang MH (2005). Pathogenicity Analysis of *Aeromonas hydrophila* on Cultured *Rana temporaria chensinensis*[J]. For. Sci.

Technol., 30(3):40-41.
Ye YW, Wang YS (1997). Operating Specification for Clinical Examination of National (2th ed). Nanjing: Southeast University Press, 553-562.
Zhang ML, Lu TY, Liu HB, Yang H (1999). Isolation and Identification on the Pathogenic Bacteria of Recessive Septicemia from Carp [J]. Chinese J. Fisheries, 12 (1):38-41.
Zhou M, Li YH, Li TY (2007). Diagnosis and Bacteria Identification about Red-leg Disease of Wood Frog [J]. Jilin Animal Husbandry Veter. Med., 6:11-13.