THE PATHOGEN OF RED BODY DISEASE IN *LITOPENAEUS VANNAMEI*

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Abstract: Red body disease occurred on a large scale in the shrimp farm in Liaoning Province in July, 2001. The characteristics of red body disease were observed by the authors. Infected animals displayed red bodies, sluggish swimming, disgusted feeding, hard shell and high mortality. Three bacterial strains were isolated from the muscles of the diseased *Litopenaeus vannamei*. In artificial infection test, one of them was proved to be the pathogen, numbered 0107. According to the traditional biochemical identification and 16S rRNA gene homology analysis, the pathogenic bacteria were *Vibrio parahaemolyticus*. Drug sensitivity test showed that the pathogenic bacteria are highly sensitive to cefoperazone, ceftriaxone, etc., while not sensitive to ampicillin and benzylpenicillin.

Key words: *Vibrio parahaemolyticus; Litolitopenaeus vannamei;* Red body disease; Pathogen

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As the selective breeding of freshwater aquaculture, *Litopenaeus vannamei* has been rapidly developed all over the country in recent years, which brings great economic and social benefits[1]. Along with the expanded scales of the shrimp breeding and the enhanced intensivism, diseases occurred frequently, such as red body disease, red leg disease, leukoderma, enteritis and gill-rot disease. Red body disease occurs frequently in some shrimp farms, the disease is commonly prevalent in the first ten days of July because of the hot weather, the fastigium of this disease will go down to the first ten days of October, when the mortality is more than 80 %. In this paper, by investigation, dissection and observed using electron microscope through negative dyeing and slice, the authors excluded the possibility of parasites and virus as the pathogens. We identified the pathogen from its physiological and biochemical characteristics and the 16S rRNA gene homology analysis; we also tested the drug sensitivity of it.

1 Materials and Methods

1.1 Shrimps Diseased shrimps (7—12cm long) were provided by Dawa Shrimp Farm in Panjin, Liaoning Province, when the fastigium of red body disease eruption. Healthy shrimps (8—11.5cm long) were provided by Zhuanghe Shrimp Farm, Liaoning Province, for the artificial infection test.

1.2 Reagents and Materials Agar medium, TCBS medium, and drug sensitive scrip were bought from Reagents Accommodate Research Center, Shanghai; PCR reagents, Taq enzyme, primers and dNTP were bought from TaKaRa Biotechnology (Dalian) Co., Ltd.

1.3 Isolation of the pathogen The bacteria were isolated from the muscles of diseased shrimps. By doing lineation on agar and TCBS media and culturing at 28 ℃ for 24h, the predominant colonies were selected to be isolated several times on agar and TCBS media until a pure culture was obtained.

1.4 Artificial infection test The healthy shrimps were selected at random and bred under the condition of the experiment for a week (9cm on average). There were 3 experimental groups and 1 control group for every bacterial strain, 10 shrimps for one group. The three bacterial strains, which had been cultured at 28 ℃ for 24h, were

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washed with 0.85 % NaCl, making the suspension of 1.2×10^9 cfu/mL. Each shrimp in the experimental groups was injected with0.1mL suspension; while those of the control groups were injected with 0.85 % NaCl at the same dose. The activity of the shrimps were recorded every 2h and the dead ones were removed until 72h later.

1.5 Modal observation and physiological and biochemical identification The modality, size and flagellum could be observed by both optical microscope and electron microscope. According to Familiar Bacteria Identification Manual[2] as well as Bergey's Manual of Determinative Bacteriology[3], the physiological and biochemical characteristics were analyzed by routine methods.

1.6 Identification of the bacteria by molecular biological methods According to the specific primers designed by Mo[4], the primers used for amplification of 16S rRNA were CTS657F: 5′-AGAGTTTGATC(C/A)TGGCTCAG-3′ and CTS657R: 5′-TACGG(C/T)TACCTTGTTACGAC TT-3′. The bacterial DNA was used as template. The reaction system was: 10 ×LA Buffer 5μL, dNTP 8μL, DNA 2μL, CTS657F (20pmoL/μL) 1μL, CTS657R (20pmoL/μL) 1μL, LATaq 0.5μL, ddH_2O 32.5μL. The reaction procedure: initial denaturation for 10min at 94 ℃; 25 cycles of denaturation (10s at 98 ℃), annealing (30s at 55 ℃) and extension (2min at 72 ℃); final extension for 5min at 72 ℃. The products were sent to TaKaRa Biotechnology (Dalian) Company for sequencing. Compared the 16S rRNA gene sequence of 0107 with those from Genbank, and obtained the phylogenetic tree by statistical and clustering analysis.

1.7 Drug sensitivity test After cultured 24h at 30 ℃, the drug sensitivity of 0107 to every kind of drug was tested by scrip. The sensitivity was identified and analyzed by ATB expression and the resistant rates were calculated. Eighteen kinds of antibiotics were used for the drug sensitivity test (Tab. 2).

2 Results

2.1 The symptoms of red body disease

The diseased shrimps first displayed sluggish swimming or held still at the bottom, sometime going round or swimming vertically. They disgusted feeding, at the same time the pleopods turned red, then the whole body, and died at last. The cuirasses and gills were light yellow, and the shells were hard. Some especial shrimps did not turn red when dying.

2.2 Characteritics

After cultured at 28 ℃on agar medium for 24 hours, the colonies of 0107 were round, translucent, smooth, and the diameter was about 2mm; while on TCBS medium, they were green and the diameter was about 3mm. The bacteria were Gram-negative, flagellate, movable and the sizes were about 0.5—0.8 ×1.4—2.6um (Fig. 1).

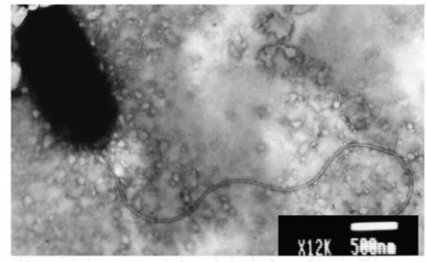

Fig. 1 Electron micrograph of the pathogenic bacteria

2.3 Results of artificial infection test

In the artificial infection test, the diseased shrimps could only be found in the experimental groups of strain 0107. Six hours later, the injected shrimps in experimental groups of strain 0107 showed different activities. They were sluggish, and most of the shrimps kept at the bottom. Eighteen hours later, several shrimps showed the symptoms of red body; 19 hours later, one shrimp died, and the others appeared red bodies in succession. The mortality was more than 50 % when 30 hours, and 72 hours later, all the shrimps in experimental groups of strain 0107 died. There were no symptoms in the shrimps from the other group. The bacteria isolated from dying shrimps under aseptic conditions were the same as the infection bacteria after identification, indicating that the isolated bacteria were the pathogens of red body disease.

2.4 Physiological and biochemical characteristics

Strain 0107 is facultative anaerobic, no special demands for nutrition, but it is sensitive to salinity, growing in 2 %—8 % NaCl, especially 2 %—3 % NaCl, but not in peptone water free from salt. It is positive to oxidase and catalase, no gas from D-glucose but acid. Tab. 1 shows the details. The results were close to the characteristics of *Vibrio parahaemolyticus* described in Bergey's Manual of Determinative Bacteriology[3], and the only differentiation was that strain 0107 can utilize melibiose

and cellobiose separately as the unique carbon source.

2.5 Results of molecular biological identification

As showed in Fig. 2, the 16S rRNA gene sequence of strain 0107 was determined as 570 bp long. The 16S rRNA sequence was homology-searched in the GenBank and compared with 100 similar sequences. In these sequences, 7 % was *Vibrio*, 92 %—99 % similarity; 9 % was *Salmonella*, 89 %—90 % similarity; 4 % was *Escherichia*, 89 %—90 % similarity; 14 % was *Pseudomonas*, 87 %—88 % similarity; 66 % was other bacteria and the unidentified bacteria, 83 %—90 % similarity. A 99 % similarity value between strain 0107 and *V. parahaemolyticus* was the highest seen among them. 27 strains were selected from 100 for the phylogenetic analysis. As showed in Fig. 3, strain 0107 had high similarities to 2 *V. parahaemolyticus* strains, indicating that they had the closest relationship.

2.6 Results of drug sensitivity test

The bacteriostasis of 18 kinds of bacteriophages to strain 0107 was detected by scrips. The relationship between the diameters of bacteriostatic circles and the sensitivities were determined by Software Whonet5.0 and ATB expression. As showed in Tab. 2, it is sensitive to 11 kinds of bacteriophages, like cefoperazone and ceftriaxone, but not to 5 other kinds, like ampicillin and benzylpenicillin.

Tab. 1 Results of physiological and biochemical test of strain 0107

Characteristics	Results	Characteristics	Results	Characteristics	Results
Gram	Negative	Gas from D-glucose	-	D-Galactose	+
Cell shape	Bacilliform	Acid from carbohydrate:		Lactose	-
Motility	+	Glucose	+	Sucrose	-
Flagellation	1 Polar lagella	D-Mannose	-	Melibiose	+
Catalase	+	D-Sorbitol	+	Cellobiose	+
Oxidase	+	Sucrose	-	D-Mannitol	+
Na^+ required for growth	+	Lactose	-	D-Sorbitol	-
Oxygen required	Facultative anaerobic	D-Galactose	+	D-gluconate	+
Reduction of No_3^- to NO_2^-	+	Trehalose	+	L-Glutamate	+
Contra-Nitration	-	D-Mannitol	+	DL-Malate	+
Amylase	+	D-Xylose	-	Citrate	+
Esculin hydrolysis	+	Arginine dihydrolase	-	Propionic acid	+
Gelatinase	+	Urease	-	Lactic acid	+
VP test	-	Indole	-	Acetic acid	+
MR test	+	Unique carbon source	for	L-Arginine	+
Growth at:		growth:		β-Alanine	-
0 % NaCl	-	Glucose	+	L-Alanine	+
5 % NaCl	+	D-Xylose	-	L-Leucine	+
8 % NaCl	+	Ribose	+	L-Serine	+
10 % NaCl	-	Trehalose	+	Ethanol	+
O/F test	Ferment	D-Mannose	+		

Tab. 2 Results of drug sensitivity test to strain 0107

Drugs	Diameters of micro-biostatic circles (mm)	Sensitivity	Drugs	Diameters of micro-biostatic circles (mm)	Sensitivity
Penicillin	10	R	Cefuroxime Sodium	16	I
Gentamicin	17	S	Erythromycin	13	R
Ciprofloxacin	25	S	Sinomin Compositea	24	S
Tetracycline	26	S	Vanoomycin	6	R
Clindamycin	11	R	Ampicillin	8	R
Aztreonam	23	S	Nitrofurantoinum	22	S
Chloramphenicol	30	S	Norfloxacin	23	S
Cefoperazone	24	S	Ceftriaxone	26	S
Ceftazidime	25	S	Amikacin	16	I

S: Sensitive I: Medial R: Resistent

GCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTATCATGAACCTTCGGGGAACGATA
ACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATT
GGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGT
CAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT
CGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTA
GCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAG

Fig. 2 Sequence of 16S rRNA gene

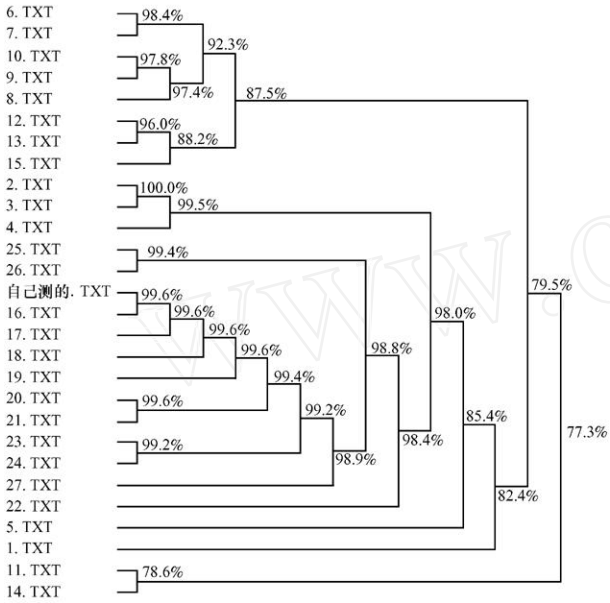

Fig. 3 Clustering result of 16S rRNA gene sequence

1. *V. parahaemolyticus* 2210633; 2. *V. vulnif icus* YJ0; 3. *V. vulnificus* CMCP6; 4. *V. vulnificus* CMCP6; 5. *V. cholerae* O1 N16961; 6. *Salmonella typhimurium* LT2; 7. *Salmonella typhimurium* DT104; 8. *Shigella flexneri*301; 9. *E. coli* O157:H7; 10. *E. coli* K12; 11. *Shewanella oneidensis* MR-1; 12. *Y. pestis* CO92; 13. *Y. pestis* KIM; 14. *P. putida* KT2440; 15. *E. chrysanthemi* 3937; 16. *V. parahaemolyticus CIP* 73.30; 17. *V. paraha emolyticus* 17802T; 18. *V. alginolyticus CIP* 70.65; 19. *V. alginolyticus* 17749T; 20. *V. campbelli* 25920T; 21. *V. harveyi* ACMM 642; 22. *V. parahaemolyticus* EcGS020801; 23. *V. fischeri isolate* 7744; 24. *V. alginolyticus* EcGS021001; 25. *V. natriegens* 14048T; 26. *V. pelagius* 25916T; 27. *V. carchiariae* 35084T

3 Discussion

Red body disease is one of the diseases which most frequently occurred on *L. vannamei* according to the reports in recent years[5—7]. There are three familiar causes for red body disease: (1) The adaptive activity of *L. vannamei* because of the mutational environment. The diseased shrimps showed red tentacles, soft shells, and red tail fins. (2) The virosis caused by *Taura Syndrome Virus* (TSV) which usually occurred one or wo two days after the sharp change of the water environment. The symptoms of *Taura Syndrome* are red tentacles and red tail fins, thus this disease is also called "red tail disease". The body turns to brown, the shell is soft and the erose black spots appear on the shell of the prolonged diseased shrimps. (3) Bacteroidal disease caused by *V. parahaemolyticus*. According to the research, it often breaks out during the last ten days of June, in the old ponds, and under the circumstances of halfway sanitization and bad water exchange. The characteristics of the disease are red appendages and at the same time the whole body turning red. Comparing with the other two reasons, the shells of dead shrimps are hard.

Strain 0107 which was isolated from the muscles of the diseased shrimps and confirmed by artificial infection test was Gram-negative and could grow on TCBS medium, showing the characteristics of *V. parahaemolyticus*. In the physiological and biochemical test, it could utilize melibiose and cellobiose as the unique carbon source, which is the only differentiation from the characteristics of *V. parahaemolyticus* described in Bergey's Manual of Determinative Bacteriology[3]. For further confirmation of strain 0107 in taxology, the amplified 16S rRNA gene sequences were determined and compared with sequences in the DNA databases of GenBank. A 99 % similarity value between strain 0107 and *V. parahaemolyticus* was the highest seen among 100 strains. Based on the physiological and biochemical characteristics as well as the 16S rRNA sequences, strain 0107 was determined to be *V.*

parahaemolyticus, which consisted with the results of Zhoug's research[8]. However, Samples in the research were from Liaoning Province and those in Zhou's paper were from Hainan Province, and the research methods are also different from Zhou's. Every taxology method has its own advantages, that is to say, wrong conclusions could be drawn if depending on only one method. So the results of multifold analysis methods are more reliable when identifying microbes.

V. parahaemolyticus, the familiar pathogen of fish, shrimps and seashells, is a kind of marine bacteria, existing widely in coasts, sediments and inside marine animals. In a general way, the disease could be avoided through improving the culturing environments and sanitizing the ponds thoroughly, because most vibrioes are conditional pathogens. Since the pathogen has already been determined to be *V. parahaemolyticus*, this article could be a good reference based on the drug sensitivity test.

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凡纳滨对虾红体病病原

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摘要:2001 年 7 月辽宁省盘锦市大洼对虾养殖厂凡纳滨对虾大面积暴发红体病。主要表现为全身发红,活动减弱,食欲减退,壳变硬,死亡率高等。本研究从患红体病的凡纳滨对虾肌肉组织中分离到三株优势菌,经回归感染证实其中一株为引发本次凡纳滨对虾红体病的病原菌,编号为 0107。对该菌进行了生理生化鉴定和 16S rRNA 基因序列同源性分析,将其判定为副溶血弧菌(*V. parahaemolyticus*)。药敏实验结果显示,该菌对头孢哌酮、头孢曲松等多种抗生素敏感,对氨苄西林、青霉素等抗生素不敏感。

关键词:副溶血弧菌;凡纳滨对虾;红体病;病原