Analysis of Antibiotic Resistant Genes in Aquacultural Bacteria

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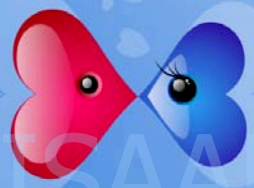
Introduction



1. present status

- **Antibiotics is the primary means for prevention and treatment of bacterial diseases in aquaculture , bacteria mutated rapidly in the selection pressure, retained the resistant strains, spread among the same or different species of bacteria in genetic level.**
- **Study confirmed that with the increase usage of drug the proportion of resistant strains also increased**

2.Potential Hazards of Resistant Bacteria

- Resistant bacteria ↑
- Resistant gene transfer among different bacteria
- Resistant bacteria transfer to human
- Antibiotic agents residue →human enterobacteria ↑
- Failure in prevention and therapy

3. Plasmid and resistance gene

Plasmid is a major medium to carry the resistance gene, in particular, the main form of transmission of bacterial resistance is the combination and transfer of resistance plasmid.

Detection of resistance genes on plasmid

1. Bacteria sensitive test

Tested bacteria (total 54 strains):

grouper (9), Trachinotus Ovatus (1), cobia (12), Oxyeleotris marmoratus (6), Channel Catfish (8), Marbled eel (3) , abalone (4), shrimp (7), Babylonia (4).

Antibiotics

- **norfloxacin, kanamycin, gentamicin, chloramphenicol, cotrimoxazole, rifampicin, furazolidone, ampicillin, ciprofloxacin, tetracycline.**

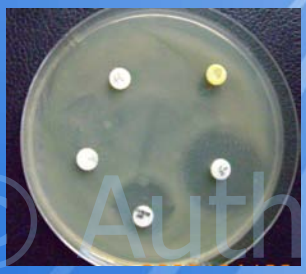
Quality control strains

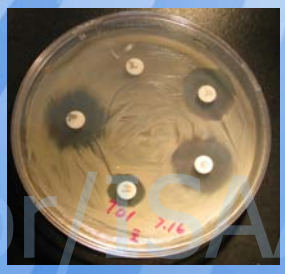
- ***Escherichia coli*** **(ATCC25922),**
- ***Staphylococcus aureaus*** **(ATCC25923)**
- **as quality control strains for laboratory preservation**

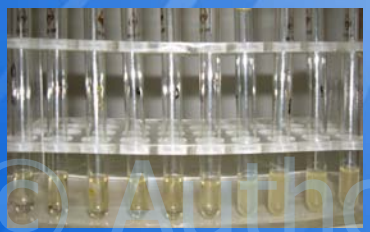
2. Sample handling

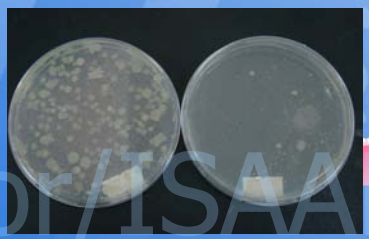
- Culture water: sulfonamides and chloramphenicol 2ppm flat. Diluted with normal saline 2 times, respectively, coated with antibiotics and antibiotic tablet.
- Animal intestine: chloramphenicol 80μg/mL, sulfonamides concentration 60μg/ml flat. grinded Animal gut, saline diluted to 10^{-3}, take 10^{-2}, 10^{-3} coated with antibiotics and antibiotic tablet.
- 28 ℃ for 72h, calculated the number of plant and drug resistant rate.

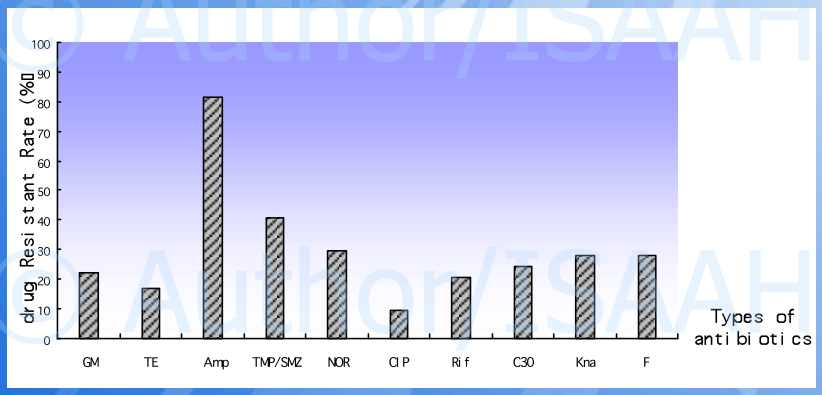
3. Results



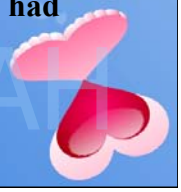








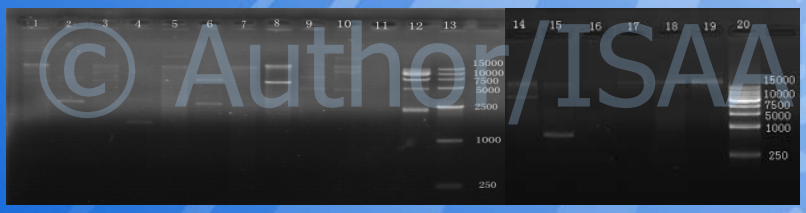
42 of the 54 gram-negative bacteria were identified as resistant bacteria (77.8%), and 37 strains (68.5%) had multi-resistances for resistance to more than 3 antibiotics.



screening of Resistance genes

1. extraction of Plasmid DNA

Plasmid extraction by alkaline extraction of plasmid kit ,There are 21 strains of resistant plasmids



1:NSV1; 2:NYV1; 3:YSL; 4:NY01; 5:NSV1； 6NSV5; 7:NBX1; 8:NB2D; 9:NTS; 10: NH T4; 11:NHS2; 12:NYL; 13:DNA mark(15000bp)

2. PCR primer

According to GenBank, registered resistance gene sequence: Su1II, cat1, cat2, cat3, cat4, gyrA, aadB, Designed the corresponding resistance gene primers :

Primer	Sequence(5'-3')	Length(bp)	Annealing temp(℃)
SulIIF	AGGGGACATGCCTGCTGAAC	20	60.67
SulIIR	CCGCAAACAGGTTACTCGCC	20	61.90
C-R	CCATCACATACTGCATGATG	20	47.20
C-1	GGTGATATGGGATAGTGTT	19	45.9
C-2	GATTGACCTGAATACCTGGAA	21	55.70
C-3	CCATACTCATCCGATATTGA	20	58.0
C-4	CCGGTAAAGCGAAATTGTAT	20	58.02
gyrAF	CCGGTACGGTAAGTTCTTCAA	21	58.01
gyrAR	GAGGAAGAGCTGAAGAGCTCCT	22	61.94
aadBF	CACGCAAGCACGATGAT	17	48.90
aadBR	TTTGTAGGCCAGTCCAC	16	50.5

Sulfonamide resistance gene Su1II, Chloramphenicol resistance gene cat1, cat2, cat3, cat4, kanamycin resistance gene aadB , were amplified as follows:

1、2、3、13、15、16、17：*Su1II*；7、9、18：*aadB*；4、8、14：*cat2*；10、11、23：*cat3*；20：*cat4*；5、6、12、19、21、22：*gyr-A*；24:Negative control；25：mark（2000bp）

The test of resistance genes

Drug Category	Sulfa	Aminoglycosides	chloramphenicol		
Resistance gene	*Su1II*	aadB	cat 2	cat 3	cat 4
Number of strains with resistance gene	9	3	3	4	1
number of Resistant strains with plasmid	18	5		11	
The detection rate of drug resistance genes	50%	60%	27%	36%	9%

Cloning and sequence analysis of Sulfonamide resistance gene Su1II of *Vibrio harveyi*

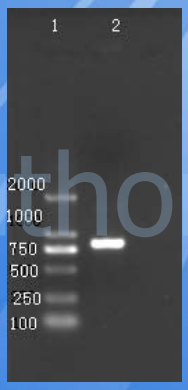
1. Primer designed

Primer was designed According to GenBank (FJ705807.1) , sequence of the Sulfonamide -resistance gene Su1II, plus restriction sites BamHI and HindIII

***Su1II*F: 5'-**
GTTGGATCCAATAAATCGCTCATCATTTTC-3'

***Su1II*R :5'-**
CCGAAGCTTGCAGTTAACGAATTCTTG-3'

2. electrophoresis results of Su1II gene PCR amplification

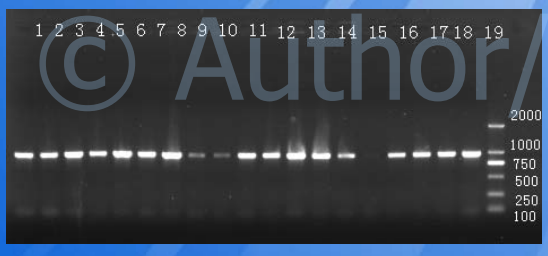


extract the plasmid DNA of *Vibrio harveyi* as a template, with designed primer to amplify Su1II gene.

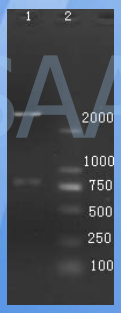
3. Cloning of PCR products and screening of positive clones

Picked at least 10 white colonies in LB liquid medium to culture 12 ~ 16h,

Identified by PCR, the result shows that specific bands.



PCR identification of recombinant plasmid electrophoresis



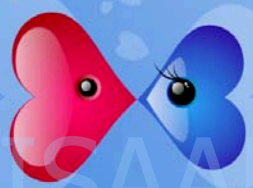
Restriction analysis of recombinant plasmid electrophoresis

Sequence analysis of cloned plasmid

- **Sequencing by the DNAstar software analysis results show that, Su1II gene with GenBank, Aeromonas hydrophila and resistant gene plasmid pRA1 Su1II sequence (FJ705807.1) were 100% homologous.**

- ***Su1II*** **‘ 582-TTCGATGAATTGCGGCTG -600’**
- **AJ289135 ‘ 582-TTCGATGAATTGCGCGTG -600’**
- **FJ705807 ‘582-TTCGATGAATTGCGGCTG -600’**

Prokaryotic expression of sulfonamide drug resistance genes Su1II of *Vibrio harveyi*



1. vector Construction and gene inserting

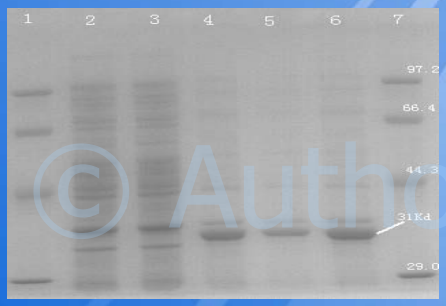
- Prokaryotic expression vector
- Su1II gene was inserted into pRSET-A prokaryotic expression plasmid, and transformed into E. coli BL21 for prokaryotic expression.

2. recombinant plasmid PCR and enzyme-digested Identification

The identification of pRSET-A- *Su1II* PCR
1:DL-2000 DNA Marker; 2-5:PCR product of recombinant plasmid

The identification of pRSET-A- Su1II Recombinant plasmid doubles enzyme-digested
1:DL-2000 DNA Marker 2,3:pET-28c(+)-Su1II Recombinnat plasmid double enzyme-digested

3. SDS-PAGE analysis of protein of Su1II



1,7:Standard protein Marker; 2,3:Induced pRSET-A（+）in control E.coli Bl21, 4~6: Protein in BL21 for inducing 2~6h

1,4,7: Standard protein Marker
2:Induced supernatant 5,6:Protein in BL21 for inducing 2~6h

Summary

- resistance rate of all the 54 bacterial was 77.8%, multi-drug resistance rate was 68.5%.
- Resistant plasmid carrying rate 50%, Su1II detection rate was 50%, aadB gene detection rate was 60%, cat2, cat3, cat4 detection rate is relatively lower.
- Su1II gene was cloned from the DNAstar software analysis published with the GenBank sequence (FJ705807.1) comparison, 100% homology, the same amino acids, are encoded resistance DHPSII.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