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研究报告

Screening of microorganisms against *Oncomelania hupensis* and assessing the molluscicidal efficacy

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Abstract: [Objective] The research aimed to screen the efficient, durable, safe and inexpensive molluscicidal microorganisms against *Oncomelania hupensis* and observe the molluscicidal effects. **[Methods]** The four strains (named as B8, B27, B36, B59) which were highly toxic to *Oncomelania hupensis* were isolated by preliminary screening from snail habitats. The colonial morphology and gram stain were observed by light microscope. The molluscicidal active fraction were analysed by different resolving gel concentration of SDS-PAGE. The ribosomal 16S rRNA of the dominant strain were amplified and the PCR products were sequenced. Sequence alignment was adopted for the identification of the dominant strain. **[Results]** The four strains were Gram-positive bacilli. According to the comprehensive comparison, molluscicidal effect of the four strains had significant differences in the fermented supernatant (FS) group (χ^2=21.286, P=0.002); And then the fermented liquor group had also significant differences (χ^2=17.298, P=0.008); The bacteria suspension group had not significant differences (χ^2=7.579, P=0.271). In addition, B59 strain showed the higher molluscicidal activity than the other three strains. The snails mortalities in FS group was up to 73.3% and 96.7% by immersion in 48 h and 72 h respectively. SDS-PAGE analysis revealed that there did not exsit protein band in FS of B59 strain and molluscicidal active components might be other materials. Finally, molecula phylogenetic analysis based on ITS sequence showed that strain B59 (accession number in GenBank: KP146144) was the most homologus to 100% *Bacillus cereus* (accession number: CP001746) with bootstrap. **[Conclusion]** In summary, The effective molluscicidal components against *Oncomelania hupensis* exsited in the fermented supernatant of strain B59, which might not be protein. B59 strain was identified as a species of *Bacillus cereus*.

Keywords: *Oncomelania hupensis*, Microorganism, Screening, Molluscicidal efficacy, Molecular phylogeny

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灭钉螺微生物的选育及抑螺效果观察

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摘 要:【目的】筛选出一种高效、持久、安全及价廉的灭螺微生物，对其进行鉴定并观察其抑螺功效。【方法】从钉螺孳生的土壤中筛选出 4 株灭螺活性较强的菌株(B8、B27、B36、B59)，显微镜观察菌株形态和革兰氏染色均为 G^+杆菌。不同分离胶浓度的 SDS-PAGE 分析其灭螺活性成分；优势菌株经 16S rRNA 扩增后，PCR 产物测序，序列比对，构建系统发育树鉴定其种属。【结果】灭螺结果表明，各试验组中发酵上清液各菌株间灭螺效果差异有统计学意义(χ^2=21.286，P=0.002)；细菌发酵液各菌株间差异也有统计学意义(χ^2=17.298，P=0.008)；菌体悬液各菌株间差异无统计学意义(χ^2=7.579，P=0.271)；此外，B59 菌株的灭螺效果优于其它菌株，尤其是其发酵上清液浸泡 48 h 和 72 h 钉螺死亡率高达 73.3%和 96.7%。SDS-PAGE 发现在 B59 细菌上清液中无蛋白带出现，推测其灭螺活性物质可能是其他成分；分子系统发育分析结果显示 B59 菌株位于 *Bacillus cereus* (CP001746)分支上，一致性达 100%。【结论】B59 菌株的发酵上清液灭螺效果最好，其灭螺活性物质可能不是蛋白质，B59 菌株被鉴定为 *Bacillus cereus*。

关键词: 钉螺，微生物，选育，灭螺效果，分子系统发育

Despite intensive long-term control programmes, schistosomiasis, a snail-transmitted parasitic disease, is still an important public health problem affecting more than 207 million people in China and Philippines, a further 6.9−7.5 million people at significant risk of infection[1-2]. But rapid re-infection easily sustains its prevalence[3]. Snail (*Oncomelania hupensis*) is the only intermediate host of *Schistosoma japonicum*[4], and an important root of schistosomiasis. Extirpation of the snail makes it possible to cut off the transmission dynamics of schistosomiasis. Snail control, therefore, is currently an important and effective prevention strategy to break the life cycle of the parasite as a major part of the National Schistosomiasis Control Programs[5-6]. However, it is still not yet clear what is the best way to treat the snail in a cost-effective way due to the complex of various impact factors, including environmental, biological and social factors. At present, the methods of suppression and eradication of snails have three kinds. Physical molluscicides are time-consuming and tough. Molluscicidal intervention in the field and labour costs make the procedure expensive and the range of application narrow. Furthermore, molluscicidal effect was slower[7]; Due to the safety concerns, high cost, environmental contamination, less selectivity, durability and the potential resistance of snail to drug and so on, chemical molluscicides are limited to the application in a large scale[8]; By contrast, biological and ecological techniques of snail control is environmental, safe and inexpensive by changing the snail breeding environment or natural enemies to break the balance of the original population. So biological control is extremely significant to environmental protection[9]. Compared with other organisms, Microbial snail control is atoxic to non-target organisms, efficient, environmental, directional and specific to *Oncomelania hupensis*. Also, microorganisms have diversity of species type, abundantly metabolic pathway (or metabolic product)[10-12], furthermore, short growth period and inducible yield ability[13]. Consequently, it is urgent to screen safe, cheap, effective microbial molluscicidal agents against *Oncomelania hupensis* and also become the main direction in the future.

Over the past decades, many researchers have been screened some molluscicidal microbes from the highly epidemic areas of snails, such as *Xanthobacter autotrophicus*, *Streptomyces violaceoruber*, *Psudomonas conrexa*, *Streptomyces griseolus* 230,

Streptomyces diiastatochromogenyes 218 and so on[14-17]. However, it is still not yet clear what are the molluscicidal active components and the mechanism of toxication. Consquently, a large variety of application effect was not ideal. Moreover, the environmental protection has currently gone from bad to worse, looking for a kind of microorganism which is harmless to humans, animals, crops and aquatic life, but virulent and effective to snail is extremely urgent. In this paper, we focused on screening toxic microbes to *Oncomelania hupensis* from the snail habitat, and finally one strain (named as B59) exhibiting highly molluscicidal activity was obtained. Moreover, the taxonomic status was determined with phylogenetic analysis. The experimental work provided a foundation for looking for the ideal microorganisms and metabolic products .

1 Materials and Methods

1.1 Snails

Adult *O*. *Hupensis* snails were collected by individual picking with forceps from the ecological experimental base of schistosomiasis prevention and control (Miluo, Hunan Province, PR. China). After feeding in the laboratory for 3 d, active adult snails with 7−8 spirals were randomly divided into groups for the molluscicidal test.

1.2 Strains

Soil samples were also taken from the same ecological experiment base. Soils were collected at about 5 cm distance to the surface. The samples were encased in sterilized kraft paper envelopes, and treated in 3 days as quickly as possible.

Strains were isolated from 15 soil samples according to the routine method. Approximately 10 g of soil was scattered in 90 mL sterile water in flasks and shaken on rotary shaker at 150 r/min for 30 min, thus forming a diluent 10^{-1}, and then the diluents were diluted 10 times in sterile water continuously until a diluent 10^{-7} was formed. Then, 0.2 mL of the diluent 10^{-5}, 10^{-6} was inoculated on the surface of LB's medium by coating respectively. They were incubated at 28 °C for 3−4 days. The well-isolated single colonies were transferred to a new LB agar[18] plate three times with an inoculating loop, then single colonies were examined with light microscope as the pure stains and stored at 4 °C finally.

1.3 Preliminary screening

The pure strains were transferred to LB liquid medium and incubated on rotary shaker at 150 r/min, 28 °C for 48 h. The suspension were diluted with dechlorinated tap water to 1% respectively. Molluscicidal activity against *O*. *hupensis* was determined with the immersion method for molluscicidal activity bioassay. At room temperature (26±1 °C), live snails were kept soaked in the test solutions from 24 h to 48 h. Suspensions in which the mortality of snails were above to 50% were chosen. The strains were stored by 20% glycerol at −20 °C until use.

1.4 Preparation of bacterial component

The strains from preliminary screening were transferred to LB medium and incubated on rotary shaker at 28 °C for 72 h. The concentration of bacterial fermented liquor (BFL) were properly adjusted to 2×10^8 bacteria/mL by hemacytometer respectively. After centrifugation, the supernatant and sediment were collected respectively. The fermented supernatant (FS) were filtrated with 0.22 μm millpore filter and stored at −20 °C until use. The sediment were diluted with sterile water and prepared to the bacterial suspension (BS). Meanwhile, the concentration of BS was regulated to be consistent with the BFL and stored at −4 °C until use.

1.5 Molluscicidal activity bioassay

Molluscicidal activity against *O*. *hupensis* was determined with the immersion method. The BFL, FS and BS were diluted 20 times with dechlorinated tap water and prepared to 5% solution (1:20, *V/V*) respectively. At room temperature (26±1 °C), three bags of live snails as one group (20 snails/bag) were submerged into flasks, which were covered with gauze to prevent snails escape, containing 500 mL of the different test solutions and were kept immersed for 24, 48 and 72 h, respectively. At the same time, dechlorinated tap water and blank medium served as two controls. Then, the test snails were removed from the bags. The snails climbing upward the wall of beakers were judged to be alive, and the snails suspected of being dead were further examined by mechanical prodding, and the snails mortality was estimated. The ratio of dead snails to total tested snails was expressed as mortality (%). Each test was

performed in triplicate on three different days, and then taken the average.

1.6 SDS-PAGE

The molluscicidal active fraction of the supernatant, after confirmed and obtained according to molluscicidal activity assay as mentioned above, was analysed by SDS-PAGE. The concentration of the stacking gel was 5%, and that of the resolving gel was 6%, 8%, 10%, 12%, 15% respectively. Finally after coomassie brilliant blue staining, the results were observed.

1.7 Statistical analysis

All datas were entered into Microsoft Excel 2010 and statistical analyses were performed using the statistical software SPSS version 18.0. A value $P<0.05$ was considered statistically significant.

1.8 Morphological observation and phylogenetic analysis

1.8.1 Morphological observation: The excellent strain obtained by molluscicidal activity bioassay was transferred to LB agar plate and incubated at 28 °C for 48 h. Then the colonial morphology was observed and gram stain was done to identify organisms with light microscope .

1.8.2 Molecular biology methods: Genomic DNA was extracted with bacterial genomic DNA extraction kit (Tiangen, China) as described by the manufacturer. The rDNA-ITS region was amplified by polymerase chain reaction (PCR). The primers P1 and P2 were designed respectively as follows: P1: 5′-AGAGTTTGATCCGGCTCAG-3′, P2: 5′-ACGGC TACCTTGTTACGACTT-3′. The 25 μL reaction mixture contained 12.5 μL 2×PCR Master Mix, 1 μL DNA template (10 mg/L), 1 μL each primer (50 μmol/L) and 9.5 μL RNase free dH_2O. The program consisted of an initial step of 5 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally extension step at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1% TAE-agarose gel. The PCR products were further purified with PCR Purification Kit (Tiangen, China), and then was sequenced by the Huada Gene Technology Co. Ltd. (Beijing, China).

The rDNA sequences were submitted to GenBank for homologue search using Nucleotide-nucleotide BLASTn programs. The reference rDNA sequence data were obtained from the GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov). The sequences were aligned with the multialignment program ClustalW[19]. A phylogenetic tree was constructed according to the neighbor joining with software MEGA version 6.0. Clade stability was assessed through bootstrap analysis using 1 000 replicates. Some known strains from the *Bacillus* MLST database were included to assess the relationships of those isolated with the ones in our study.

2 Results

2.1 Molluscicidal activity of the strains screened from the soils

A total of 96 pure strains were isolated from the snail habitat. After primary screening, four strains which had higher molluscicidal activity on snails were picked up and named B8, B27, B36 and B59. By gram stain, these strains were Gram-positive bacteria in light microscope.

The four strains were employed to test the molluscicidal efficacy. All three different bacterial components groups of the four strains against snails within a 24, 48 and 72 h exposure period was different. Generally, the snail mortality was increased with the increase of molluscicide treatment time (Table 1).

On the whole, the fermented supernatant showed better molluscicidal effect (P=0.002). The mortalities of snails were above to 65% for 48 h and 90% for 72 h; Secondly, the snails mortalities of the bacterial fermented liquor were above to 50% and 95% for 48 and 72 h respectively (P=0.008); Finally, the bacterial suspension showed the worst molluscicidal effect (P=0.271). The mortalities of snails were low to 50% and 80% for 48 and 72 h. In addition, the four strains also showed different virulence. B59 strain had stronger molluscicidal activity than the other three strains. Particularly, The snails mortalities in FS group reached ro 73.3% and 96.7% by immersion in 48 and 72 h respectively; B27 was the second. The snails mortalities of FS group reached to 70% and 90% by immersion in 48 h and 72 h; the last was B8 and B36. So B59 was decided as the proper strain for subsequent experiments.

2.2 SDS-PAGE

The result of SDS-PAGE analysis revealed that there did not exsit protein bands in the FS of B59 strain. This showed that other chemical components

Table 1 Total mortality of snails immersed in bacteria components at different time
表 1 分离菌株各成分浸泡不同时间钉螺死亡情况

Group 试验组	Strain 菌株	24 h		48 h		72 h		Whole test 整体检验
		No. dead snails 死螺数	Mortality 死亡率 (%)	No. dead snails 死螺数	Mortality 死亡率 (%)	No. dead snails 死螺数	Mortality 死亡率 (%)	
BFL	B8	3	5.00	31	51.7	58	96.70	χ^2=17.298
	B27	15	25.00	49	65.0	57	96.70	P=0.008
	B36	17	28.30	46	76.7	57	96.70	
	B59	15	25.00	46	76.7	55	91.70	
FS	B8	5	8.33	40	66.7	58	96.70	χ^2=21.286
	B27	10	16.70	42	70.0	54	90.00	P=0.002
	B36	7	11.70	39	65.0	55	91.70	
	B59	22	36.70	44	73.3	58	96.70	
BS	B8	2	3.33	10	16.7	16	26.70	χ^2=7.579
	B27	5	8.33	28	46.7	36	60.00	P=0.271
	B36	5	8.33	20	33.3	45	75.00	
	B59	10	16.70	34	56.7	46	76.70	
Medium control	B8	2	3.33	27	45.0	56	93.30	χ^2=11.565
	B27	5	8.33	30	50.0	58	96.70	P=0.072
	B36	7	11.70	23	38.3	56	93.30	
	B59	10	16.70	33	55.0	56	93.30	
Dechlorinous water control	B8	0	0	0	0	4	6.67	
	B27	0	0	0	0	1	1.67	
	B36	0	0	0	0	5	8.33	
	B59	0	0	0	0	6	10.00	

might play a role in killing snails, but not some proteins. The specifically molluscicidal active fractions are needed further research.

2.3 Identification and phylogenetic date analysis of the dominant strain

The colony morphology of the four strains were circular, smooth, opalescent and opaque. They were Gram-positive bacilli. By agarose gel electrophoresis, PCR product of B59 strain was a 1 500 bp (Figure 1), and with the accession number KP146144 assigned by GenBank. With the addition of sequences from GenBank, the phylogenetic tree was constructed by the neighbour joining method using *Escherichia coli* as the outgroup strain. The overall structure of the maximum-likelihood tree generated from concatenated sequences resulted in two phylogenetic groups. But they had higher homology between the two groups (100%) (Figure 2). Accordingly, the rDNA-ITS sequence analysis indicated that B59 strain was identified as a species of *Bacillus cereus*.

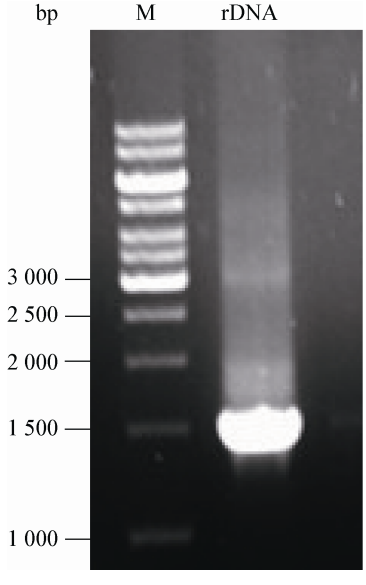

Figure 1 Electrophoretogram of B59 strain 16S rRNA based on product by PCR
图 1 B59 菌株 16S rRNA PCR 产物扩增的电泳图谱

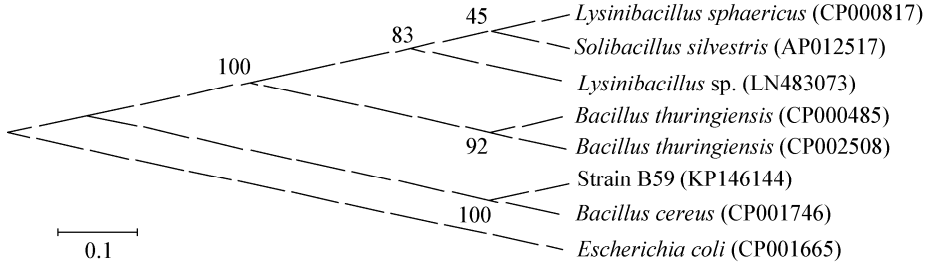

Figure 2 Phylogenetic trees of B59 strain based on rDNA-1TS gene sequence analysis

图 2 B59 菌株基于 rDNA-1TS 序列的系统发育树

Note: Accession numbers are shown in parenthese. All the reference sequences comes from GenBank database. Scale bar represents the nucleotide substitution percentage. Bootstrap values are shown at each node.

注：括号中数字表示 GenBank 序列号，参比序列均来自 GenBank 库. 标尺代表核酸置换率，每个节点上数字表示自举值.

3 Discussion

Here we have reported that four strains with better molluscicidal activity were isolated from the snails habitat. Four strains and their metabolic products on snails toxicity test showed that the fermented supernatant had better molluscicidal activity. The snails mortalities in the fermented supernatant were above to 65% and 90% in the case of submerged period 48 h and 72 h respectively, which showed that the effective components of killing snails were mainly existed in the fermented supernatant. The result was consistent with Guo et al. research[20]. But there were not protein bands in the FS of B59 strain by SDS-PAGE. The lethal effect of the active substance on snails might be more related to energy metabolism or toxin[21]. The specifically molluscicidal active fractions are worth further studying.

Then, stain B59 was identified as a strain of *Bacillus cereus* by phylogenetic analysis. To our knowledge, *B*. *Cereus*, Gram-positive spore-forming bacteria, could produce several compounds (degradation enzymes, cytotoxic factors and cell-surface proteins) that might contribute to virulence[22-24]. The illnesses associated with *Bacillus cereus* are probably mediated by the synergistic effects of a number of virulence products. These products, known to accumulate only during stationary phase when high bacterial densities are reached, included a various haemolysins/cytolysins, two enterotoxic complexes, non-haemolytic enterotoxinand[25]. However, it is rare saying that *B*. *Cereus* possesses molluscicidal activity.

In summary, the established snails-killing strategy described here using *B*. *cereus*, has advantages of rapid treatment, easy operation, atoxic to non-target organisms and good anti-*O*. *hupensis* effects, it is suitable for the emergency treatment and long term planning of infected areas. The strain B59 as candicate molluscicide is promising and practical application value to provide continuous and environmentally safe molluscicidal effect. Our present work is to further optimize the molluscicide condition, to make clear the specific substance which played an important role in killing snails in the supernatant. This would help to eliminate or inhibit *O*. *hupensis* to a large extent.

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