Original Article

New Nodule Type Found in the Lungs of Pomacea canaliculata, an Intermediate Host of Angiostrongylus cantonensis

*Yue GUO 1, Hong Chang ZHOU 1, Ying DONG 1, Ting ZHANG 1, Yu Yang SUN 1, Jian Feng ZHONG 2, Yu Liang CAO 3, Sheng Wen SHAO 1, Yong Liang PAN 1, Hai Yan DONG 1

1. School of Medicine, Huzhou University, Huzhou, China
2. Infectious Diseases Dept., Huzhou Central Hospital, Huzhou, China
3. Intensive Medicine, No.98 Hospital of PLA, Huzhou, Zhejiang, China

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Abstract
Background: Pomacea canaliculata (P. canaliculata) lung nodules, were commonly caused by Angiostrongylus cantonensis infection. Here, we found a new nodule type without any parasites.

Methods: Overall, 447 P. canaliculata snails were collected in Ning Bo, Zhe Jiang, China in 2018. In order to exhibit the similarities and differences between two nodules types (2018, Huzhou Zhejiang, China), both types were collected in formalin for tissue pathological sectioning. Besides, to obtain the microbial community of the new nodule, the 18S ribosomal RNA (rRNA) gene of it was amplified and analyzed using the Illumina second-generation sequencing platform.

Results: Although two nodules were found in the lungs of P. canaliculata, they were different in shape and pathology. Illumina sequencing indicated Poterioochromonas sp., a species of golden algae, might be the causing agent of the new nodule.

Conclusion: We firstly found a new pathological nodule type in the lungs of P. canaliculata, and this nodule might be induced by golden algae infection, however, the direct link between the golden algae and the new nodules, as well as the nodules’ impact on the snails’ physiology and A. cantonensis infection require further study.

Keywords:
Pomacea canaliculata, Lung nodule, 18S ribosomal RNA, Poterioochromonas sp. Angiostrongylus cantonensis

*Correspondence Email: guoyue66@126.com

Introduction

The freshwater snail, Pomacea canaliculata is a globally invasive species (1). Wide-ly distributed in southeast China, this snail has a bad reputation as the intermediate host of Angiostrongylus cantonensis, the causative agent of eosinophilic meningitis (2). In China, P. canaliculata is frequently mistaken for Cipangopaludina chinensis Gray, a local edible snail and
not a natural vector for *A. cantonensis*. This mistake directly induces many cases of cosinophilic meningitis in China.

*Angiostrongylus cantonensis* infection occurs in many *P. canaliculata* organs, including the foot and lung sac (3). *A. cantonensis* infection can leave pathological nodules in the snail host’s lungs. Microscopic detection of larvae nodules in the lungs of *A. cantonensis* -positive snails is the primary pathological sign of *A. cantonensis* infection. These signs are used to characterize *A. cantonensis* infections because it is cheap and efficient (4). Here, we found a new nodule type without any parasites.

**Materials and Methods**

**Snail samples and nodule collection**

Overall, 447 *P. canaliculata* snails were collected in Ning Bo, Zhe Jiang, China in 2018. After the shell was broken, the snail’s mantle skirt was cut from the body and its lung sac was cut open and flattened in 0.6% physiological saline under a microscope. The prevalence of both the *A. cantonensis*-induced nodules and the new nodules was recorded. Lung sacs with *A. cantonensis*-induced nodules and new nodules were collected in formalin and used for tissue sectioning, followed by hematoxylin and eosin (H&E) staining. Photographs were taken microscopically. The new nodule surfaces were then microscopically cut with a scalpel, gently squeezed with eye forceps and internal substances were collected in 0.6% physiological saline for sequencing.

**DNA extraction and amplification**

DNA from the new nodules was extracted using the Magic Mag Micro Genomic DNA Extraction Kit (NO. B518749, Sangon Biotech Co., Ltd. Shanghai, China). Both bacterial 16S rRNA and eukaryotic 18S rRNA were amplified by PCR and sequenced by the Illumina MiSeq platform (Origin-gene Biomedical Technology Co., Ltd., Shanghai, China). The primer information is listed in Table 1; the first primer was used to amplify the bacterial 16S rRNA gene (5-7), and the second was used to amplify the V4 region of the eukaryotic 18S rRNA gene (8, 9).

A TransStart FastPfu DNA Polymerase 20-μl reaction system was used in the ABI Gene-Amp® 9700, including 4 μl of 5×FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8μl of forwarding primer (5 μM), 0.8μl of reverse primer (5 μM), 0.4μl of FastPfu Polymerase, and 10ng of template DNA. PCR parameters were as follows: an initial denaturation step at 95 °C for 5 min, 27 cycles at 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec, with a final extension step at 72 °C for 10 min.

**Table 1: Primer Information**

| Primers         | Primer Sequences                  |
|-----------------|-----------------------------------|
| Primer 1        | 341F 5’-CCTAYGGGRGBCASCAG-3’      |
| 806R            | GGAACNNGGATCTAAT-3’               |
| Primer 2        | TAReruk454FWD1 5’- CCAGCASCYGCCTATCC-3’ |
| TARerukREV3     | 5’- ACTTCTGGTCTTTGATYRA-3’        |

PCR products were detected by 0.2% agarose gel electrophoresis and purified by the AxyPrep DNA Gel Extraction Kit (AXYGEN Biosciences). Quanti Fluor™ST was then used to quantify the PCR products, and DNA was pooled to construct an Illumina library after Illumina PE250 sequencing (10-12).

**Data and taxonomic analyses**

Raw data provided by the PE reads from the Illumina PE 250 were stitched together by the following software: Trimmomatic (13), FLASH (14), Usearch (15), and QIIME (16). The raw data were denoised, trimmed, quality-filtered, and aligned to construct the operational taxonomic unit (OTU) matrix.

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
OTU information was then used to classify groups of closely related individuals.

To reveal the community composition per sample, representative OUT sequences with similar levels above 97% were taxonomically analyzed by RDP Classifier, Bayesian algorithm. QIIME and RDP Classifier (ver. 2.2) were applied for taxonomic research (17-19).

Results

Pathological description

Nodules in the *P. canaliculata* snail lung sac included two types: *A. cantonensis*-induced nodules and the newly discovered nodules (Fig. 1).

The *A. cantonensis*-induced nodules contained 2nd- or 3rd-stage *A. cantonensis* larvae. When the nodule was torn open, *A. cantonensis* larvae could be detected microscopically.

The new nodules were the latest discovery, first exhibited here. These nodules were shaped like poached eggs, with two different-sized globes set together. The smaller globe was composed of cells and the larger globe was transparent, surrounding the smaller one to form a poached-egg shape microscopically. The new nodules differed in size, with the smallest being 0.1 mm in diameter, and the biggest being 2 mm in diameter. Both microscopic detection and tissue sectioning showed that these nodules were not parasite larvae (Fig.1).

Size differences among the new nodules suggested that they were developing and might be caused by micro-organisms infection or cancerous tissues. The new nodules were either masses of exogenous cells or snail cells (Table 2).

![Fig.1](image)

**Table 2:** Differences and similarities between two lung nodule types in *Pomacea canaliculata*

| Nodule description | *A. cantonensis*-induced nodules | Newly discovered nodules |
|--------------------|----------------------------------|--------------------------|
| Parasite           | All nodules contain curled larvae| No parasites inside      |
| Size               | Mostly similar in size: 0.25±0.02mm | Different sizes ranging from 0.1 to 2mm |
| Shape              | Smaller spherical, with no transparent surrounding material | Poached-egg shape, with transparent material surrounding it |
| Color              | Dark and bright regions          | Even colored             |
| Location           | Lung                             | Lung                     |
| No. positive snails/total number (rate) | 4/447 (0.9%) | 14/447 (3%) |

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Prevalence of the two nodule types in P. canaliculata

Among the 447 P. canaliculata snails collected, four were A. cantonensis-infected (0.9% infection rate), showing A. cantonensis-induced nodules in the lung sacs, and 14 contained the new nodules (3% infection rate). The new nodules’ prevalence was much higher than that of the A. cantonensis-induced nodules.

Microbial community composition in the new nodules

The bacterial 16S rRNA gene of the newly discovered nodules was amplified by PCR, and the results exhibited no positive bands on electrophoresis (Fig.2A), indicating that the new nodules might not be caused by bacterial infection. Conversely, the new nodules’ eukaryotic 18S rRNA gene was successfully amplified, which suggested these nodules were eukaryotic (Fig.2B).

Overall sequence descriptions

Overall, 43491 raw sequences were obtained from the new nodules, 40959(94.18%) of which ranged from 351-400bp (base pairs).

Per the OTU analysis, the compositions of the new nodules were as follows. The dominant phyla were Streptophyta (34.5%), Chordata (26.01%), Arthropoda (1.77%) and Ascomycota (1.16%) (Fig. 3A). The dominant genera were Poterioochromonas (30.7%) and Gladiolus (25.27%) (Fig. 3B), and at the species level, Poterioochromonas sp. might be the causative pathological agent of the new nodules.

Discussion

Nodules in snail hosts are common pathological structures (20, 21). In Biomphalaria glabrata, a novel bacterial pathogen-induced pathological nodules, widely distributed in the snail’s mantle, hepatopancreas, and ovotestis, leading to increased mortality (22). In Bulinus jousseaumei, another bacterial pathogen caused nodules in superficial areas of the snail’s body, such as the pseudo branch, foot, collar, mantle, and tentacles (23). However, this has rarely been reported in P. canaliculata snails.

In addition, since P. canaliculata widely inhabit the freshwater system in tropical and subtropical zones where microorganisms are rich, this snail often forms facultative and obligate symbiotic associations with bacteria or viruses.
biodiversity in these nodules, but thus far, only limited knowledge of these nodules exists. The interconnection between the new nodules and the snail host, its physiological impact on the snail, and the new nodules’ formation require further study.

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Conflict of interest

The authors declare that there is no conflict of interests.

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