SCREENING FOR NEW NEMATOCIDAL SUBSTANCES OF MICROBIAL ORIGIN BY A NEW METHOD USING THE PINE WOOD NEMATODE

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Parasitic diseases are widespread, currently affecting about 3×10^9 people and innumerable domestic animals.[1] Nematodes (round worms) are a group of most important parasitic organisms. Many attempts have been made to find potent nematocidal substances, however, only a few antibiotics are available in practice.[2]

Recently, avermectins, which have a potent nematocidal activity and are used in the veterinary fields, were discovered by the *in vivo* screening method using mice infected with *Nematospiroides dubius*.[3,4] KIMURA *et al.*[5] devised a method to distinguish alive nematodes from dead ones, which is based on the nature of living nematodes that descend through Japanese paper. We attempted to develop a screening method for nematocidal antibiotics using the pine wood nematode *Bursaphelenchus lignicolus* by KIMURA's method with modifications. Consequently, a more rapid and simple method was successfully established and then used for practical screening work.

The present paper deals with the new screening method and the results of screening for nematocidal antibiotics among various known antibiotics of our laboratory's stock and from soil isolates.

The pine wood nematode *B. lignicolus* was grown for about 10 days on slants of *Botrytis cinerea* growning on potato - glucose agar medium. The growing nematodes (propagative form) were harvested from slants by the Baermann funnel technique, counted under the microscope, and suspended in an appropriate volume of distilled water to contain a definite number of nematodes (*ca.* 500 nematodes/ml). An immersion test was carried out using a test tube (1×10.5 cm). To 0.1 ml of the nematode suspension (*ca.* 50 nematodes) in a test tube were added 0.3 ml of a cultured broth of a soil isolate or 10% acetone solution of purified material and 0.1 ml of streptomycin solution (500 μg/ml). The top of the tube was sealed with Japanese paper (Kokuyo type writing paper, Tai-19). After incubation for 18 hours at 27°C, the tube was placed upside down in a well of a tissue culture plate (Nunc, 24-wells) containing 1 ml of streptomycin solution (100 μg/ml), and allowed to stand for 3 more hours at 27°C. Then, the number of nematodes in the well which descended through Japanese paper was counted under the microscope. The nematocidal activity was expressed as mortality:

$$\text{Mortality (\%)} = \left\{1-\frac{A+(B\times0.5)+(C\times0.25)}{D}\right\}\times100$$

where A is the number of nematodes with active motility, B is the number of nematodes with moderate motility, C is the number of nematodes with weak motility, and D is the number of living nematodes in the control.

Fig. 1 summarized the method using *B. lignicolus* for *in vitro* nematocidal activity. The method is more simple than KIMURA's assay because it uses a small test tube and a 24-well microplate and as a result saves assay time; it takes about 24 hours while the KIMURA's method needs 4 days. The IC_{50} of avermectin B_{1a} assayed by our method was 0.1 μg/ml.

We tested the nematocidal activities of 163 known antibiotics from our stock using the above method. Among them, colistin A, polymyxin B_1, gramicidin S, antimycin A, siccanin, staurosporine[6] and setamycin[7,8] in addition to avermectin were found to possess significantly strong nematocidal activities against *B. lignicolus* (Table 1). The nematocidal activities of the above antibiotics other than antimycin A have never been known although that of antimycin A against *Rhabditis* sp. has been reported.[9] The IC_{50} values of polypeptide antibiotics colistin A, polymyxin B_1, and gramicidin S were significantly small.

Next, broth filtrates of about 10,000 strains of actinomycete soil isolates were screened for nematocidal activity using the above method. Consequently, two new antibiotics, jietacins A and B were discovered.

Fig. 1. A new screening method for nematocidal substances.

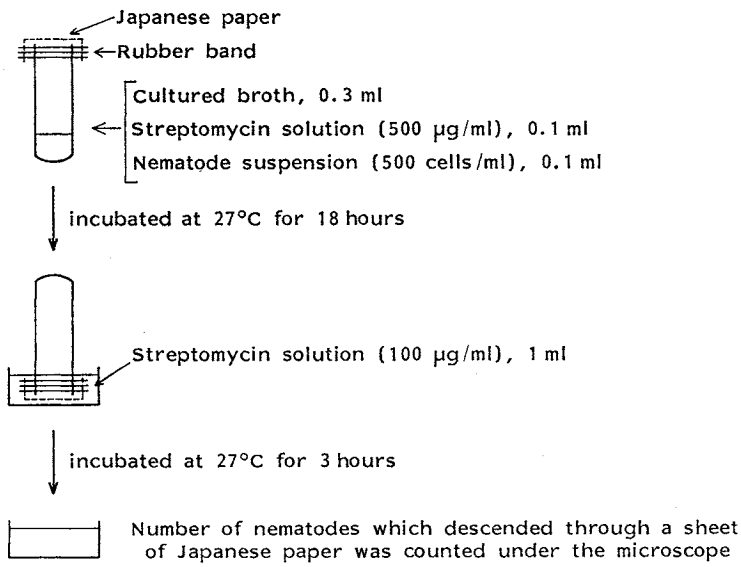

Table 1. Nematocidal activities (IC_{50} values) of some known antibiotics against *Bursaphelenchus lignicolus*.

Compound	IC_{50} (μg/ml)
Colistin A	1.0
Polymyxin B_1	1.0
Gramicidin S	0.1
Antimycin A	6.0
Siccanin	6.0
Staurosporine	3.5
Setamycin	10
Avermectin B_{1a}	0.1

The isolation, physico-chemical and biological properties of the new nematocidal antibiotics as well as the taxonomy of the producing strain have been reported by the authors.[10] These are three times as active (IC_{50} 0.038 μg/ml) against *B. lignicolus* as avermectin B_{1a}. It is considered from the physico-chemical properties that they have an azoxy group in their structure. A large amount of jietacin A was prepared and submitted for toxicity evaluation. When it was administered intraperitoneally to mice at the dose of 200 mg/kg, no acute toxicity was observed.

In this screening work, 5 preparations from soil isolates exhibiting a relatively strong nematocidal activity were identified as known antibiotics, antimycin A, aureothin, neoaureothin, leucanicidin and piericidin A. Table 2 shows

Table 2. Nematocidal activities of piericidin A, aureothin, neoaureothin and leucanicidin, which were identified in our screening work from soil isolates, against *Bursaphelenchus lignicolus*.

Compound	IC_{50} (μg/ml)
Piericidin A	5.0
Aureothin	0.5
Neoaureothin	0.25
Leucanicidin	10

their nematocidal activities (that of antimycin A appears in Table 1). Among the antibiotics identified, aureothin and neoaureothin exhibited strong nematocidal activities. These antibiotics inhibited completely the nematode at 6 times higher concentrations than their IC_{50} values. Aureothin was discovered as a byproduct of aureothricin production from *Streptomyces thioluteus*[11] and later was reported to possess nematocidal activity against *Rhabditis* sp.[9] Neoaureothin[12] (spectinabillin)[13] was discovered also as a byproduct of neoantimycin[12] or streptovaricin[13] production by *Streptoverticillium orinoci* or *Streptomyces spectabilis*, respectively. The nematocidal activities of neoaureothin, leucanicidin and piericidin A are reported for the first time.

From the above results, our new screening method described above proves to be useful for searching new nematocidal antibiotics.

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