

**Guidelines**  
**for**  
**Safety Evaluation of Microbial Pesticides**  
**(Draft Translation)**

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## **I. Standards for safety evaluation of microbial pesticides**

The present standards introduce basic approaches for safety evaluation of microbial pesticides, and specify date which shall be submitted by a registration applicant for safety evaluation.

### **1. Basic approaches for safety evaluation**

(1) In the present standards, "microbial pesticides" shall mean pesticides containing virus, bacterium, fungus, protozoan or nematode (limited to those containing like as the symbiotic bacterium as active ingredient) in a viable state which are manufactured or imported for sale for pesticidal purpose. Microbe-derived pesticides which are natural enemies to parasitic bees, predatory insects, etc. and antibiotics do not come under the category of microbial pesticides.

Genetically modified microbes are not included in it either for the moment.

(2) Safety in humans and effects on the organisms etc. in the environment, the safety shall be demonstrated or confirmed on the basis of scientific data shown in paragraph 2 below.

(3) Toxicological studies for safety evaluation shall be performed in compliance with the GLP standard notified by "Notification concerning standards for proper toxicological studies on Agricultural Chemicals (Notification 59 Nohsan No. 3850 \* dated August 10, 1984), of Director-General of Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries)."

(4) If, as a result of after evaluation based on the data submitted, it becomes necessary to prove or confirm the safety from the standpoint of biological properties of the microbe and method of using the microbial pesticide, the applicant shall be required to submit additional data.

### **2. Data serving as the basis for safety evaluation**

(1) Data necessary for safety evaluation of microbial pesticides are stipulated for in the Attached List "Data required, by type of microbe, for safety evaluation of microbial pesticides."

(2) When the data for safety evaluation have already been submitted for the registration application of other microbial pesticides formulation, the said data can be used for the registration application of new formulation. In addition, when one applicant agrees that other applicant uses the said data, which have already been submitted for registration, new applicant is required to submit a certificate indicating that the said data is allowed to use by an former applicant.

(\* ) the notification was replaced to Notification 11 Nohsan No.6283 dated October 1,1999

Attached List

**Data required, by type of microbe, for safety evaluation of microbial pesticides**

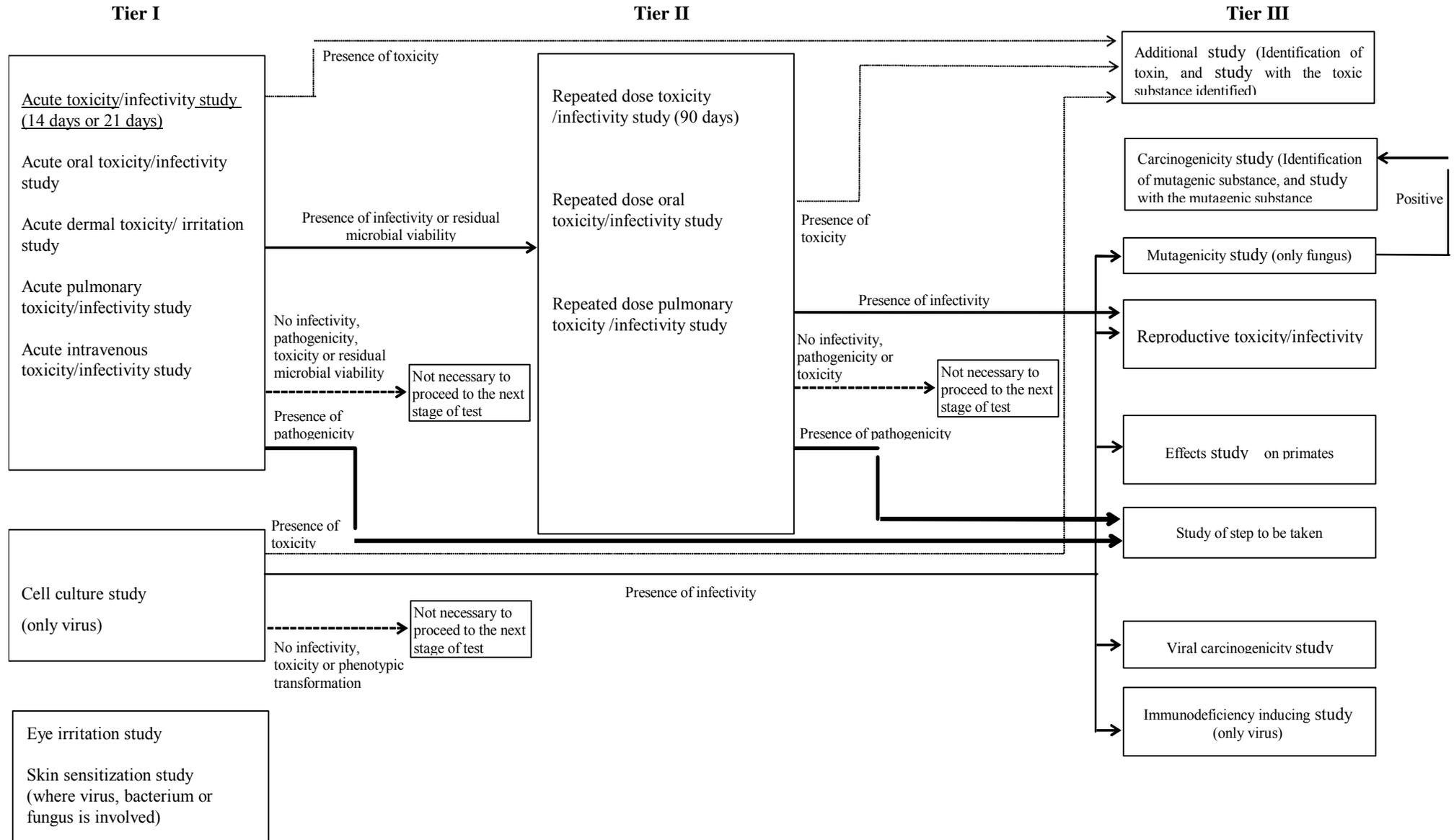
Data required	Type of microbe		Remarks
	Virus	Others	
1. Data concerning specification and properties of microbial pesticides			
(1) Name and taxonomic position of microbe	○	○	
(2) Biological properties of microbe	○	○	
(3) Composition of technical grade of the active ingredient	○	○	
(4) Production method for technical grade of the active ingredient	○	○	
(5) Composition of end-use product	○	○	
(6) Production method for end-use product	○	○	
2. Data concerning method of use			
(1) Diseases, pests and weeds for which the microbial pesticide is to be used, and crop plants to which the microbial pesticide is to be applied	○	○	
(2) Method of use and use level	○	○	
3. Results of studies of safety in humans			
(1) Acute oral toxicity/infectivity study	○	○	
(2) Acute dermal toxicity/ irritation study	○	○	
(3) Acute pulmonary toxicity /infectivity study	○	○	
(4) Acute intravenous toxicity/infectivity study	○	○	
(5) Eye irritation study	○	○	
(6) Skin sensitization study	○	○	Test should be performed where virus, bacterium or fungus is involved.
(7) Cell culture study	○	×	
(8) Repeated dose toxicity /infectivity study	□	□	This test should be performed if any infectivity or residual microbial viability has been found in the acute toxicity/infectivity study
(9) Mutagenicity study	×	▲	If, in the case of fungi, any infectivity has been observed in the repeated dose toxicity /infectivity study, this test should be performed.
(10) Reproductive toxicity/infectivity study	▲	▲	If the repeated dose toxicity /infectivity study has revealed any infectivity etc. this test should be performed.
(11) Viral carcinogenicity study	▲	▲	If any infectivity etc. , has been noted in mammalian animal cells in the cell culture study, this study should be performed.
(12) Immunodeficiency inducing study	▲	×	If the cell culture study has revealed any infectivity etc. in mammalian animal cells, this study should be performed.
(13) Effect test on primates	▲	×	- ditto -
4. Data concerning cases of hypersensitive reactions that occurred in production and use of microbial pesticides	○	○	

Data required	Type of microbe		Remarks
	Virus	Others	
5. Results of studies of residual microbial viability on crop plants	<input type="checkbox"/>	<input type="checkbox"/>	If, in the case of microbial pesticides for food crops, any effects have been observed in the Tier I of safety in humans, excluding primary eye irritation and dermal sensitization studies, this test should be performed.
6. Results of effect test on the organisms in the environment			
(1) Toxicity study on freshwater fish	<input type="radio"/>	<input type="radio"/>	When there is no possibility of exposure, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.
(2) Toxicity study on freshwater invertebrates	<input type="radio"/>	<input type="radio"/>	- ditto -
(3) Avian toxicity study	<input type="radio"/>	<input type="radio"/>	- ditto -
(4) Study of effects on non-target plants	<input type="radio"/>	<input type="radio"/>	- ditto -
(5) Study of effects on non-target insects	<input type="radio"/>	<input type="radio"/>	- ditto -
(6) Study of effects on honey bees	<input type="radio"/>	<input type="radio"/>	- ditto -
(7) Study of effects on silkworms	<input type="radio"/>	<input type="radio"/>	- ditto -
(8) Study of effects on soil microbes	<input type="radio"/>	<input type="radio"/>	- ditto -
7. Results of studies of fate in the environment	<input type="checkbox"/>	<input type="checkbox"/>	If any effects have been observed in the effect test on the organisms in the environment, required tests should be performed.
○: Required in the Tier I □: Required in the Tier II ▲: Required in the Tier III ×: Not required			

(Note) As regards the Tier I, II or III, if any infectivity etc. have been found in the Tier I in any of the tests, the test proceeds to the Tier II, and Tier III of test is performed if any infectivity etc. have been observed in any of the Tier II or if any infectivity etc. have been noted in the Tier I of the cell culture test. Proceeding of the test stage in studies of safety in humans is shown in Attached Fig. 1, and effect test on the organisms in the environment (Tier I) and studies of fate in the environment (Tier II) are shown in Attached Fig. 2.

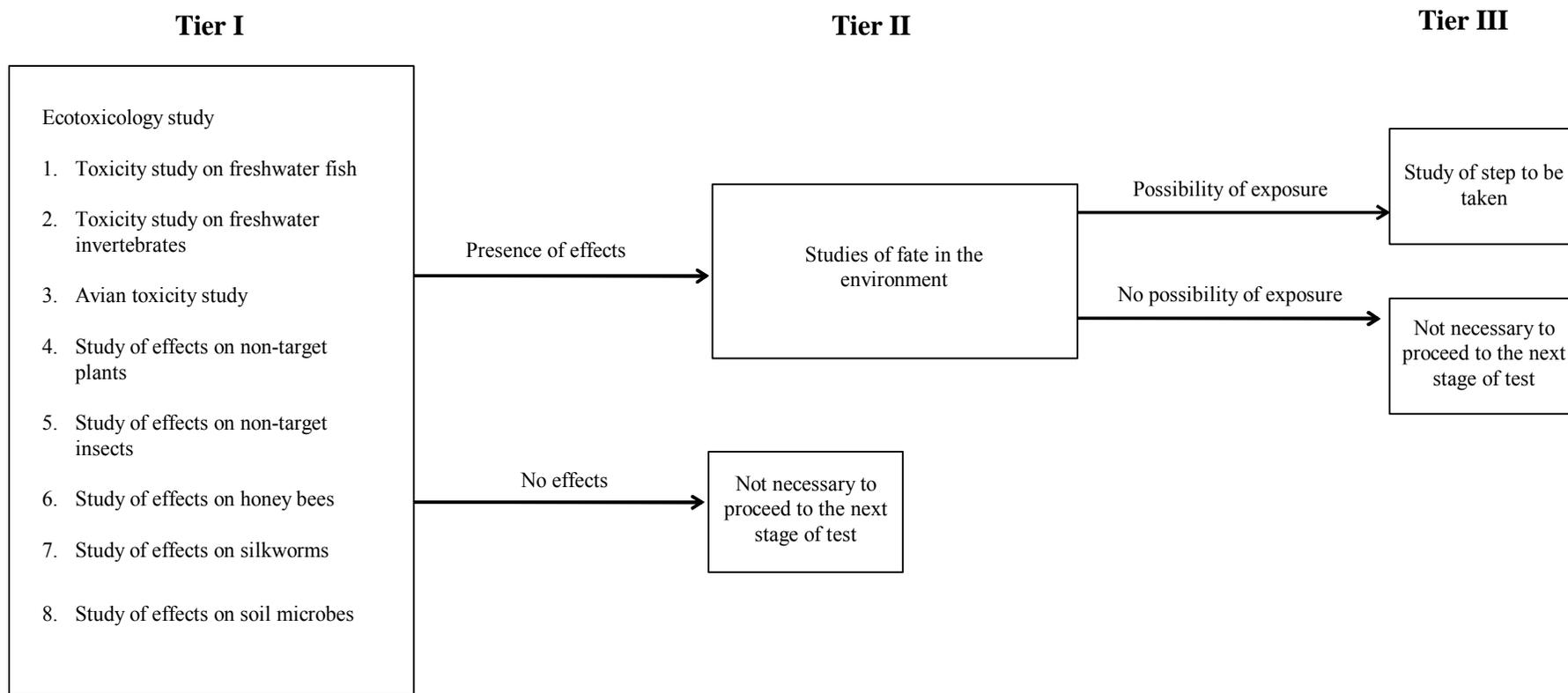
Attached Fig. 1

### Toxicological studies for safety evaluation



Attached Fig. 2

### Ecotoxicology study and Environmental fate study



(Note) Where there is no possibility of exposure, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, test may be omitted.

## **II. Guidelines for preparation of data necessary for safety evaluation of microbial pesticides**

### **1. Basic stance on interpretation of these guidelines**

- (1) These guidelines are intended for use as guiding rules for preparation of the data necessary for safety evaluation which are required in application for registration of microbial pesticides.
- (2) Testers are not required to strictly comply with these guidelines in conducting necessary tests. The present guidelines do not preclude maintaining certain flexibility by making some change or improvement in the test methods according to the characteristics of the test substance, so as to better meet the purposes of the test.

### **2. Test substance**

- (1) Where technical grade of the active ingredient is used as test substance
  - a. When technical grade of the active ingredient is used as test substance, its composition should be the same as that of the technical grade used for the commercially available end-use product of the microbial pesticide.
  - b. When it is difficult to use technical grade of the active ingredient as test substance, a microbe which is the same in form (trophozoite, spore, crystal, etc.) as the microbe contained in the technical grade of the active ingredient for the commercially available end-use product may be used instead of the technical grade.
- (2) Where end-use product is used as test substance
  - a. When an end-use product is used as test substance, a product form considered to be most frequently used should be selected, as a rule, from the standpoint of ensuring safety in actual use of the microbial pesticide.
  - b. Where it is difficult to use an end-use product as test substance, technical grade of its active ingredient may be used instead of the end-use product.
- (3) As a rule, the test substances used should be of the same lot throughout the test period. When, under unavoidable circumstances, test substance of another lot has to be used, it should be sufficiently close to the previous lot in content and composition. The lot number(s) of the test substance used should be stated in the record of the test results.
- (4) The content and composition of each test substance used in safety evaluation tests should be mentioned in the record of the results of each test.
- (5) Units of test substances
  - a. For bacterial vegetative cells, CFU (colony forming unit) should be used as unit.
  - b. In the case of protozoa and embedded viruses, hemocytometer should be used for counting.
  - c. In the case of end-use product containing spores of bacterium or fungus, counting should be made on the basis of CFU or with the use of hemocytometer.
  - e. For end-use products containing mycelium of fungus, dry weight of  $10^{-9}$  g should be used as unit.
  - f. As regards units for other items, quantitative determination should be made using the most suitable method according to the type of the microbe concerned.

### **3. Test animals and plants**

From the standpoint of accurate safety evaluation of microbial pesticides, animals or plants of the same species and strain which were raised or grown under the same condition and are in healthy condition should be used for each test item. Also in selecting test animals or plants for test of effects on the living things in the environment, careful selection should be made, taking into consideration such factors as possibility of exposure judging from the type of pesticidal microbe, method of using the microbial pesticide and place of use in addition to other living things closely related to the target living thing. Reason for selection should be stated.

### **4. Confirmation and detection of pesticidal microbe**

For confirmation and detection of the microbe used as active ingredient (hereinafter referred to as "pesticidal microbe") in a microbial pesticide, a method high in selectivity, sensitivity and reliability should be used according to the type of the microbe concerned.

### **5. Definition of terms**

In these guidelines, the under mentioned terms have the following meanings:

(1) Infectivity

When a pesticidal microbe, after its invasion into the test animal or plant or into the cultured cell, has proliferated, the pesticidal microbe is said to have infectivity.

(2) Pathogenicity

When a pesticidal microbe has caused infection in the test animal or plant, resulting in development of a disease in the said animal or plant on the cellular tissue level or on the individual whole body level, the pesticidal microbe is said to have pathogenicity.

(3) Toxicity

Although a pesticidal microbe has not caused any infection in the test animal or plant, nor in the cultured cell, the toxin or poison produced by the said microbe or the base material used for proliferation of the said microbe has brought about some reaction detrimental (harmful to life) to the said test animal or plant or to the said test cultured cell.

In such a case, the pesticidal microbe is said to have toxicity.

(4) Residual microbial viability

Although a pesticidal microbe has not caused any infection in the test animal or plant, it remains, without dying, in or on the animal, plant or in the soil in a viable state for a certain period of time after application. In such a case, the pesticidal microbe is said to have residual viability.

## **III. Data concerning specification and properties of microbial pesticides**

### **1. Name and taxonomic position of microbe**

(1) Name of microbe

Scientific name (synonym), and name in Japanese and English

(2) Taxonomic position of microbe

Class, order, family, genus, species, subspecies, line, serotype, strain, etc.

- (3) Methods for isolation and identification (morphological, biochemical and serological methods, etc.)
- (4) Origin (including artificial treatment, genetic homeostasis, etc.)

## **2. Biological properties of microbe**

- (1) Growing condition (temperature, pH, nutrition, etc.)
- (2) Range of hosts, history of life, mechanism of action
- (3) Presence of toxin, its production and properties, and method for its identification
- (4) Presence in the natural world, and geographical distribution
- (5) Known and potential harmfulness to humans, environment and non-target living things, and discussion thereof

## **3. Content and composition of technical grade of the active ingredient**

Content (potency) of microbe, and type, content and properties of additive(s)

## **4. Production method for technical grade of the active ingredient**

Production method (including composition of culture medium, culturing condition and refining method) and method for quality control [method for securing microbial identity and content (potency), method for preventing biological contaminants from entering the product, method for storing and preserving foundation stock (strain) and technical grade material of the active ingredient, and so forth]

## **5. Composition of end-use product**

Content (potency) of microbe, and type and content of other ingredient(s)

## **6. Production method for end-use product**

Production method, and method for quality control (method for securing uniformity and stability of the product)

# **IV. Toxicological studies for safety evaluation**

## **1. Acute oral toxicity /infectivity study**

### **(1) Objective**

To evaluate the toxicity/infectivity in humans of the microbial pesticide with oral exposure, the pesticidal microbe at a high concentration level should be administered orally to the test animals, in a single dose and evaluation should be made of its clinical and pathological effects on the test animals, including death.

### **(2) Test method**

- a. Test substance: Technical grade of the active ingredient
- b. Test animals: Rats or mice (SPF, ; young adults; females to be nulliparous and non-pregnant; range of body weight to be within mean value  $\pm 20\%$ ; and animals to be fasted for a certain period before and after administration)
- c. Establishing test groups:  
Control group:

Vehicle control group (2 or more animals/sex)

Non-administration group (2 or more animals/sex. This group is needed where the toxicity of the vehicle is unknown.)

Dosage group:

$10^8$  units/animal (1 ml or less/100 g of body weight: a single oral dose)

Final sacrifice Group (5 or more animals/sex)

Interim sacrifice Groups (3 or more animals/sex/group, animals to be sacrificed at Day 3, 7 and 14 after administration (to examine changes in residual microbial viability with lapse of time).

d. Test period

As a rule, Test period should be for 21 days after administration.

e. Observation and examination items

(a) Observation of symptoms

Observation period should be made, usually for 21 days, as to type, severity, onset and course of development of symptoms, and to reversibility of such symptoms in relation to time. The results of the observation should be recorded.

(b) Measurement of body weight

Individual weights of animals should be determined shortly before administration, and at Day 7, 14 after administration, and at death and sacrifice.

(c) Excreting out of pesticidal microbe

Number of microbe in feces should be made regularly (before administration and at Day 1, 3, 7 and 14 after administration).

(d) Necropsy

When an animal dies during test period, it should be necropsied immediately, and the date of death and findings from necropsy should be recorded. Surviving animals are to be sacrificed for necropsy, at the end of the test.

As regards interim sacrifice groups, 3 or more animals each of male and female should be sacrificed at Day 3, 7 and 14 after administration. Presence of infection etc. in organs should be examined, and findings should be recorded together with the date of necropsy.

(e) Residual microbial viability in the body

In the necropsied animals, number of microbe should be made in the kidneys, brain, liver, lungs, spleen, stomach, blood, small intestine, large intestine and major lymph nodes, and in each of the organs where macroscopic lesions were observed.

(3) Summarization of the results

Based on the results of observation of symptoms, necropsy and microbial count, a summary should be made for each of the following items:  Clinical signs and symptoms  Mortality  Body weight changes  Excreting out of the pesticidal microbe  Pathological changes  Presence of infection by organ

(4) Proceeding to the next stage of test

a. When none of the infectivity, pathogenicity, toxicity and residual microbial viability have been observed in the test animals, no further studies are required.

b. When any infectivity or residual microbial viability has been seen in the test animals, repeated dose oral toxicity /infectivity study (90 days) should be performed.

- c. When any pathogenicity has been found in the test animals, a further study should be performed after enough considerations.
- d. When any toxicity has been noted in the test animals, the toxic substance should be identified, and toxicological studies of the toxic substance identified, should be performed based on the “Guidance on toxicology study Date for Application of Agricultural Chemical Registration” (Notification 59 Nohsan 4200, dated January 28, 1985, of Director - General of Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

## 2. Acute dermal toxicity / irritation study

### (1) Objective

To evaluate the toxic effect in humans of the microbial pesticide with dermal exposure, the pesticidal microbe at a high concentration level should be applied once to the skin of the test animals, and local irritation to and systemic effect on the test animals should be examined.

### (2) Test method

a. Test substance: Formulation

b. Test animals

Albino rabbits (SPF, ; young adults; females to be nulliparous and non-pregnant, and range of body weight to be within mean value  $\pm$  20%)

c. Establishing test groups

Control group:

Unnecessary (When the toxicity of a vehicle used in the administration is unknown, vehicle control group and Non- treatment group (2 animals/sex, in each group) are necessary.)

Treatment group:

$10^8$  units/animal (5 or more animals/sex, 2 g or less/animal: 24hours before application, Fur from 10% or more of the body surface area, should be shaved, and test substance should be applied, then the application site should be covered with gauze patch. The test substance should be removed with water or an appropriate s vehicle at the end of a 24 hours application period.)

d. Test period

As a rule, test period should be for 14 days after administration

e. Observation and examination items

(a) Observation of symptoms

Observation period should be made, usually for 14 days, as to type, severity, onset and course of development of symptoms, and to reversibility of such symptoms in relation to time. The results of the observaton should be recorded.

(b) Measurement of body weight

Individual weights of animals should be determined shortly before test substance application, weekly thereafter, and at death and sacrifice.

(c) Necropsy

When an animal dies during test period, it should be necropsied immediately. And surviving animals should be sacrificed for necropsy at the end of the test. Records should be made of the date of death, date of necropsy in addition to the findings from necropsy.

(d) Status of dermal irritation

Dermal reaction should be examined immediately after application and every day thereafter for symptoms of erythema and edema, and dermal irritation is rated and recorded, according to the criteria in Table 2 below.

(3) Summarization of the results

Based on the results of the observation of symptoms and the necropsy, a summary should be made for each of the following items:  Clinical signs and symptoms  Mortality  Body weight changes  Pathological changes  Status of dermal irritation (according to the criteria of irritation in Table 2).

(4) Proceeding to the next stage of test

- a. When no toxicity has been observed in the test animals, no further studies are required.
- b. When any toxicity has been found in the test animals, the toxic substance should be identified, and toxicological studies of the toxic substance identified should be performed based on “Guidance on Toxicology Study Data for Application of Agriculture Chemical Registration” (Notification 59 Nohsan 4200, dated January 28, 1985, of Director-General of Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

Table 2. Skin irritation and corrosion criteria

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Erythema and eschar formation	
No erythema.....	0
Very slight erythema (barely perceptible) .....	1
Well defined clear erythema .....	2
Moderate to severe erythema .....	3
Severe erythema (deep redness) or eschar formation (Erythema is not scorable) .....	4
Maximum score: 4	
Edema formation	
No edema .....	0
Very slight edema (barely perceptible).....	1
Slight edema (edges of area well defined by definite raising) .....	2
Moderate edema (swelling of approximately 1 mm).....	3
Severe edema (swelling $\geq$ 1 mm, and extending Beyond the exposed area) .....	4
Maximum score: 4	

---

**3. Acute inhalation toxicity/infectivity study**

(1) Objective

To evaluate the toxic effect in humans of the microbial pesticide with exposure, through the respiratory system, the pesticidal microbe at a high concentration level should be exposed to the test animals through the respiratory tract in a single dose, and evaluation should be made of its clinical and pathological effects on the test animals, including death.

(2) Test method

a. Test substance: Technical grade active ingredient

b. Test animals

Rats or mice (SPF, ; young adults; females to be nulliparous and non-pregnant; and range of body weight to be within mean value  $\pm$  20%)

c. Establishing test groups

Control group:

Vehicle control group (2 or more animals/sex)

Non-administration group (2 or more animals/sex.

This group is needed where the toxicity of the vehicle is unknown.)

Dosage group:

$10^8$  units/animal (0.3 ml or less/100 g of body weight: a single dose through the respiratory tract)

Group final sacrifice (5 or more animals/sex)

Groups interim sacrifice (3 or more animals/sex/group, animals to be sacrificed immediately after administration, and at 3Day, 7 and 14after administration, to examine changes in residual microbial viability with lapse of time).

d. Test period

As a rule, Test period should be for 21 days after administration

e. Observation and examination items

(a) Observation of symptoms

Observation period should be made, usually for 21 days, as to type, severity, onset and course of development of symptoms, and to reversibility of such symptoms in relation to time. The results of the observation should be recorded.

(b) Measurement of body weight

Individual weights of animals should be determined shortly before administration, and at Day 7 and 14 after administration, and death and sacrifice.

(c) Necropsy

When an animal dies during test period, it should be necropsied immediately, and the date of death and findings from necropsy should be recorded. Surviving animals are to be sacrificed for necropsy, at the end of the test.

As regards interim sacrifice groups, 3 or more animals each of male and female should be sacrificed immediately after administration, and at Day 3, 7 and 14 after administration. In each necropsy, presence of infection etc. in organs should be examined, and findings should be recorded together with the date of necropsy.

(d) Residual microbial viability in the body

In the necropsied animals, number of microbe should be made in the lungs, nasal cavities and tracheae in addition to counting in the kidneys, brain, liver, spleen, blood and major lymph nodes, and also in organs where macroscopic lesions were observed.

(3) Summarization of the results

This should be done according to (3), 1. of Section IV (with the exception of □ - Excreting out of the pesticidal microbe).

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section IV.

#### **4. Acute intravenous toxicity/infectivity study**

##### **(1) Objective**

To evaluate the toxicity and infectivity that direct invasion of the microbial pesticide into the human body may have on the health, a single dose of the pesticidal microbe at a high concentration should be administered to the animals intravenously, and evaluation should be made of its clinical and pathological effects on the test animals, including death.

##### **(2) Test method**

- a. Test substance: Technical grade active ingredient
- b. Test animals: Rats or mice (SPF, ; young adults; females to be nulliparous and non-pregnant; and range of body weight to be within mean value  $\pm 20\%$ )
- c. Establishing test groups:

Control group:

Vehicle control group (2 or more animals/sex)

Non-administration group ( 2 or more animals/sex.

This group is needed where the toxicity of the vehicle is unknown.)

Dosage group:

$10^7$  units/animal ( 0.3 ml or less/100 g of body weight: a single intravenous dose)

Group final sacrifice (5 or more animals/sex)

Groups interim sacrifice (3 or more animals/sex/group, animals to be sacrificed immediately after administration, and at Day 3, 7 and 14 after administration, to examine changes in residual microbial viability with lapse of time).

- d. Test period

As a rule, Test period should be for 21 days after administration

- e. Observation and examination items

- (a) Observation of symptoms

Observation period should be made, usually for 21 days, as to type, severity, onset and course of development of symptoms, and to reversibility of such symptoms in relation to time. The results of the observation should be recorded.

- (b) Measurement of body weight

Individual weights of animals should be determined shortly before administration, and at Day 7 and 14 after administration, and at death and sacrifice.

- (c) Necropsy

When an animal dies during test period, it should be necropsied immediately, and the date of death and findings from necropsy should be recorded. Surviving animals are to be sacrificed for necropsy, at the end of the test.

As regards interim sacrifice groups, 3 or more animals each of male and female should be sacrificed immediately after administration, and at Day 3, 7 and 14 after administration.

In each necropsy, presence of infection etc. in organs should be examined, and findings should be recorded together with the date of necropsy.

- (d) Residual microbial viability in the body

In the necropsied animals, a number of microbes should be made in the blood, kidneys, brain, liver, spleen, small intestine, large intestine and major lymph nodes, and in organs where macroscopic lesions were observed.

(3) Summarization of the results

This should be done according to (3), 1. of Section IV (with the exception of □ - Excreting out of the pesticidal microbe).

(4) Proceeding to the next stage of test

- a. When neither pathogenicity nor toxicity has been observed in the test animals, no further studies are required.
- b. When any pathogenicity has been found in the test animals, a careful study should be made concerning further study to be made.
- c. When any toxicity has been noted in the test animals, the toxic substance should be identified, and toxicological studies of the toxic substance identified, should be performed based on "Guidance on toxicology study Data for Application of Agricultural Chemicals Registration" (Notification 59 Nohsan 4200, dated January 28, 1985, of the Director - General of Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

## 5. Eye irritation study

(1) Objective

To estimate the eye irritation in humans of the microbial pesticide coming into contact with the eyes, the microbial pesticide should be applied to the eyes of the animals, and then eye irritation should be examined.

(2) Test method

- a. Test substance: Formulation
- b. Test animals  
Albino rabbits (SPF, ; young adults, females to be nulliparous and non-pregnant, and range of body weight to be within mean value  $\pm 20\%$ )
- c. Establishing test groups:  
Control group:  
Vehicle control group (unnecessary: the eye on one side is to be used as control)  
Non-treatment group (3 or more animals. This group is needed where the toxicity of the vehicle is unknown.)  
Treatment group:  
 $10^7$  units/eye (6 or more animals.  
0.1ml/eye for testing liquids, 0.1g/eye for testing solids: It should be ascertained within 24 hours before treatment that the eyes have no abnormalities. When the treatment of test substance is likely to cause any pain, it may be given under local anesthetic 24 hours after treatment, the test substance applied should be washed off with lukewarm water.)
- d. Test period  
As a rule, Test period should be for 7 days after exposure
- e. Observation items

(a) Observation of symptoms

The eyes should be examined according to the criteria in Table 1 below:

Observation of irritation symptom and injury to the eye should be made 1 hour after administration, and 1, 2, 3, 4 and 7 days after treatment

If, on the 7th day, any irritation symptom is observed, follow-up observation should be made every 3 days thereafter, for up to the maximum of 21 days

(4) Summarization of the results

Based on the results of observation, a summary should be made for each of the following:

- Scores for eye irritation should be counted according to the criteria in Table 1.
- Severity of irritation and erosion
- Occurrence of any disorders other than those of the eye

When the pesticide is dropped on the surface of the eye, a reversible change that may result is called eye irritation, while irreversible change is called eye erosion.

(5) Proceeding to the next stage of test

This is not particularly required.

Table 1. Eye irritation and corrosion criteria

---

1. Cornea	
A. Opacity : Degree of opacity (determined according to the most opaque area)	grade
No ulcers or opacity observed.....	0
Sporadic or diffused opacity (different from the degree of cloudiness having ordinary luster); the details of the iris are clearly translucent.....	1
There are some clear areas left, but nearly all of the iris is obscured.....	2
Nacreous area, no details of iris visible, size of pupil barely discernible.....	3
Corneal opacity; iris not discernible through opaque areas.....	4
B. Area of corneal opacity	
None.....	0
0 - 1/4.....	1
1/4 - 1/2.....	2
1/2 - 3/4.....	3
>3/4.....	4
Injury value = A × B × 5 (Maximum value 80)	
2. Iris	
A. Lesion	grade
Normal.....	0
Clear and deep frugae, congestion, swelling, moderate hyperemia in the corneal periphery; any of these singly or in combination; the iris still reacts to light (reaction is slow and dull).....	1
No reaction to light; hemorrhage; gross destruction (any or all of these).....	2
Injury value = A × 5 (Maximum value 10)	

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3. Conjunctiva	grade
A. Redness (eyelid, bulbar conjunctiva, cornea, and/or iris)	
Blood vessels are normal .....	0
Clear hyperemia in some blood vessels .....	1
Diffuse crimson; individual blood vessels cannot be readily discerned .....	2
Diffuse beefy red .....	3
B. Conjunctival edema (palpebral conjunctiva and/or nictitating membrane)	
	grade
No swelling .....	0
Greater than normal swelling (including nictitating membranes) .....	1
Obvious swelling accompanying ectropion of eyelids .....	2
Swelling such that the eyelid less than half .....	3
Swelling such that the eyelid is half or more .....	4
C. Exudation	
No exudation .....	0
Slight abnormal liquid discharge (different from inner canthus discharge of normal animals) .....	1
Eyelids, adjoining skin and fur are wet with abnormal liquid discharge. ....	2
Because of large amount of liquid discharge, larger areas of eyelids, adjoining skin and fur are wet. ....	3

---


$$\text{Injury value} = (A + B + C) \times 2 \quad (\text{Maximum value } 20)$$


---

Total maximum injury value =  
injury value of cornea + injury value of iris + injury value of conjunctiva

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## 6. Skin sensitization study

### (1) Objective

To estimate the sensitization that may be caused by repeated exposure to the microbial pesticide with administration through the human's skin or respiratory tract, the microbial pesticide should be treated to the test animals by intradermal injection, and the sensitization thus caused should be examined.

This test should be performed when pesticidal microbe involved is virus, bacterium or fungus.

### (2) Test method

a. Test substance: Formulation

b. Test animals

White guinea pigs (SPF, ; young adults, males)

c. Establishing test groups:

Control group:

    Positive control group (5 or more animals, well-known allergen)

    Treatment group:

10 or more animals

When the test substance is in a liquid, it should be used undiluted. When in a solid, it should be used after being diluted with saline. (When the test substance is a skin irritant, it should be used after its irritating property has been weakened with saline.)

After removal of fur from the test animals, the first induction exposure is done at 0.05 ml/animal, with subsequent exposure to be done every other day at 0.1 ml/animal for 3 weeks, totaling 10 times of intradermal injection, are done for induction 2 weeks after the 10th injection, a challenge exposure is done.

d. Test period

For a period from the start of exposure to 48 hours after challenge injection

e. Observation items

At 24 and 48 hours after challenge injection, observation should be made of erythema, edema and other reactions.

(3) Summarization of the results

Reactions observed after administration should be recorded.

(4) Proceeding to the next stage of test

Not particularly required.

7. **Cell culture study**

(1) Objective

To examine whether a microbial pesticide containing a virus as active ingredient has infectivity or toxicity, and whether causes phenotypic transformation, tests should be performed by applying mammalian cells with the virus.

(2) Test method

a. Test substance

In a case of occluded form virus, non-occluded virus should be used. In a case of other types of virus, virus extracted from infected host cells or tissues should be used. The unit of virus to which the test substance is exposed should be plaque-forming unit (PFU). Where it is impossible to perform plaque assay, LD50 on value host insects or TCID50 on value sensitive cells should be used as unit. Concerning insect viruses, the test substances should be free from blood and lymph of the insect except where it has been confirmed that these contaminants have no effect on the cell culture.

b. Cell strain

As a general rule, the following cell strains should be used: Primary culture of human embryonic cell Human diploid cell strain Primate-derived established cell line Primary culture of Syrian hamster embryonic cell (SHE) Sensitive cell strains that can be used for proliferation and quantitative determination of the virus concerned.

c. Observation items

(a) Infectivity test

More than  $10^6$  units of virus should be inoculated to subconfluent (about  $2 \times 10^5$  cells/ petri dish )Each cell strain

In this test, at least 5 PFUs of virus or 7 units of virus in terms of LD50 should be used per cell. When a unit smaller than this is used, reason for it should be stated.

For control, non-inoculation control group, inactivated control group and positive control group should be set. Equal volume of the medium for invertebrate animal cells used in virus culture are added to non-inoculation control group. Inactivated virus is inoculated to inactivation control group. Sensitive cells or host cells are used in positive control group.

Cells are subcultured on 7 and 14 days after inoculation, and observation should be made for a total of 21 days to see if any cell denaturation occurs.

The culture medium should be recovered, for quantitative determination of infectious virus by using adequate host system, 1, 2, 5, 7, 14 and 21 days after inoculation; and quantitative determination should be made of viral antigen in the cell and of nucleic acids.

(b) Cytotoxicity test

Each cell strains shown in □ to □ above should be inoculated into 30 petri dishes at 200 cells per petri dish, and after 24 hours, 10 of the petri dishes should be inoculated with virus at  $10^5$  unit/plate. To another 10 petri dishes, non-inoculation control, should be added equal volume of the medium for invertebrate animal cells used in viral solution, and the remaining 10 dishes should be used as non-treated control which is added equal volume of the medium for vertebrate animal cells.

After 1 hour exposure, all petri dishes should be washed with the medium for vertebrate cells, and incubation should be performed for a period of time during which a small colony consisted of more than 25 cells forms in the non-treated control, and then, observation should be made of the effect on the colony formation of the cell strain in each of the 3 groups.

(c) Cell phenotypic transformation test

This test is not required where viral nucleic acids has not been confirmed in the cell in infectivity test.

SHE cell should be inoculated with virus, and observation should be made of the occurrence of any phenotypic transformation. For control, the following groups should be set: non-inoculation group which is added only the medium for invertebrate animal cells, inactivated control group which is inoculated with inactivated virus, and positive control group [a group in which SHE cell is inoculated with simian adenovirus Type 7 (SAV-7)].

When any phenotypic transformation has been observed, the cell in the colony with phenotypic transformation should be inoculated into the hamster to observe tumor formation.

(3) Summarization of the results

Based on the results of the observation, a summary should be made for the following items:

- Presence or absence of cell denaturation
- Results of the quantitative determination of infectious virus
- Effect on colony formation
- Presence or absence of phenotypic transformation
- Presence or absence of tumor formation in the case where phenotypic transformation has been observed

- (4) Proceeding to the next stage of test
- a. When no infectivity, toxicity nor phenotypic transformation has been observed in the mammalian cells, no further cell culture studies are required.
  - b. When any infectivity has been seen in any of the mammalian cells, viral carcinogenicity study, Reproductionve toxicity/infectivity study , immunodeficiency inducing study , and test of effects on primates should be performed.
  - d. When any toxicity has been noted in mammalian cells, the toxic substance should be identified, and using the toxic substance identified, a test should be performed based on the "Notification concerning handling of the results of toxicity tests performed in connection with application for registration of agricultural chemicals" (Notification No. 59 Nosan 4200, dated January 28, 1985, of the Director of Plant Protection Division, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

## **8. Repeated dose toxicity/infectivity study (90 days)**

### (1) Objective

When any infectivity or residual microbial viability has been observed in the single dose test, the pesticidal microbe in a high concentration should be given to the animals once every day for a period of 90 or more days, so as to evaluate the effect of the repeated intake of the pesticidal microbe.

### (2) Test method

#### a. Test substance

The same test substance as used in the acute toxicity/infectivity study should be used.

#### b. Test animals

The same animals as used in the acute toxicity/infectivity study should be used.

#### c. Establishing test groups

Control group:

Vehicle control group (10 or more animals/sex)

Non-administration group (10 or more animals/sex.

This group is needed where the toxicity of the vehicle is unknown.)

Dosage group:

$10^8$  unit/animal (10 or more animals/sex, through the administration route in which infectivity or residual microbial viability was observed in the single dose test)

#### d. Test period

As a rule, Test period should be for 90 days after administration

#### e. Observation and examination items

##### (a) Observation of symptoms

Observation period should be made, usually for 90 days, as to type, severity, onset and course of development of symptoms, and to reversibility of such symptoms in relation to time. The results of the observation should be recorded.

In addition, food consumption in each week should be recorded.

##### (b) Measurement of body weight

The body should be weighed shortly before administration, and every week thereafter, and at the time of death and sacrificing.

##### (c) Necropsy

When an animal dies in the course of test, it should be necropsied immediately, and the date of death and findings from necropsy should be recorded. Surviving animals are to be sacrificed for necropsy, at the end of the test.

In each necropsy, the date of necropsy and findings should be recorded, including the presence of any infection in the organs.

(d) Residual viability of pesticidal microbe in the body

In the necropsied animals, number of microbe should be made in the kidneys, brain, liver, lungs, spleen, stomach, blood, small intestine, large intestine and major lymph nodes, and in each of the organs where macroscopic lesions were observed.

(3) Summarization of the results

Based on the results of observation of symptoms, necropsy and microbial count, a summary should be made of the following:  Clinical signs and symptoms  Mortality  Food consumption  Changes in body weight  Pathological changes  Presence of infection by organ

(4) Proceeding to the next stage of test

- a. When none of the infectivity, pathogenicity and toxicity have been observed in the test animals, no further tests are required. However, for any infectivity found in the single dose test, the reason should be made clear.
- b. When any infectivity has been seen in the test animals, Reproductive toxicity/infectivity study should be performed. When the pesticidal microbe involved is a fungus, a mutagenicity study should also be conducted.
- c. When any pathogenicity has been noted in the test animals, a careful study should be made concerning further test to be made.
- d. When any toxicity has been found in the test animals, the toxic substance should be identified, and using the toxic substance identified, a test should be performed based on the "Notification concerning handling of the results of toxicity tests performed in connection with application for registration of agricultural chemicals" (Notification No. 59 Nosan 4200, dated January 28, 1985, of the Director of Plant Protection Division, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

## 9. Mutagenicity study

(1) Objective

When a microbial pesticide containing a fungus as active ingredient has shown any infectivity in the repeated dose study, a mutagenicity study should be performed to evaluate whether there is any risk of carcinogenicity.

(2) Test method

- a. Test substance: Extract from the technical grade of active ingredient (liposoluble substance)
- b. Test items  
The following three tests should be performed:
  - (a) Reverse mutation study with bacteria
  - (b) Chromosomal aberration study with cultured mammalian cells
  - (c) Micronucleus study (with rodents)

(3) Summarization of the results

A summary should be made of the results. Where considered necessary, a dose-response curve should be attached to it.

(4) Proceeding to the next stage of test

When a test has shown a positive result, the mutagenic substance should be identified, and using the mutagenic substance identified, a carcinogenicity test should be performed based on the carcinogenicity test shown in the "Notification concerning handling of the results of toxicity tests performed in connection with application for registration of agricultural chemicals" (Notification No. 59 Nosan 4200, dated January 28, 1985, of the Director of Plant Protection Division, Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries).

## 10. Reproductive toxicity/infectivity study

(1) Objective

To evaluate the effect of a microbial pesticide on the reproduction performance of mammals and on fetal growth in addition to evaluation of its transfer into neonates, a reproduction study should be conducted in any of the following cases by giving the pesticidal microbe to both males and females before mating, and further by continuing administration to females after successful pregnancy, so as to examine the effect of the microbial pesticide on the reproduction and fetuses, including transfer of the microbe into the neonates.

- When any infectivity was observed in the test animals in the repeated dose study.
- When the pesticidal microbe concerned is considered to be closely related to the microbes known to parasitize mammalian cells.
- Where a pesticidal microbe is not sufficiently isolated and refined, with a resultant possibility that the pesticidal microbe may contain microbial contaminants that parasitize mammals.
- When the pesticidal microbe concerned is a virus, and it showed infectivity in mammalian cells in the cell culture study.

(2) Test method

a. Test substance: Technical grade active ingredient

b. Test animals

Rats or mice (SPF, ; 6 - 8 weeks old; males: 20 or more animals, females: enough number to obtain 20 or more pregnant animals, avoid those with a low fertility.)

c. Establishing test groups

Control group:

Vehicle control group

Non-administration group (This group is needed where the effect of the vehicle is unknown.)

Dosage group:

$10^8$  unit/animal (1 ml or less/100 g of body weight: oral administration. Where effect was seen only in administration through the respiratory tract in the acute toxicity/infectivity study, the test substance should be given through the said route at  $10^8$  unit/animal (0.3 ml or less/100 g of body weight). It should be given 2 weeks before mating, and during mating period and pregnancy with such frequency that moderate to high level of infection can be maintained.)

- d. Test period  
From start of the administration to sacrifice of the neonates
- e. Observation and examination items
  - (a) Observation of symptoms  
Observation period should be made, usually for a period from start of the administration to sacrificing of the neonates, as to type, severity, onset and course of development, and to reversibility of such symptoms in relation to time. The results of the observation should be recorded.  
For pregnant females, such items should be observed and recorded as daily food consumption, course of pregnancy, and prolongation of gestation period, if any. As regards neonates immediately after birth, such items should be observed and recorded as body weight of the neonates, the number per litter, number of stillborns offspring, number of live offspring, appearance of neonates, including external abnormalities etc.
  - (b) Measurement of body weight  
The body should be weighed just before administration, thereafter every week, and at the time of death and sacrifice.
  - (c) Necropsy  
The male animal should be sacrificed as soon as the pregnancy of its pairing female has been confirmed.  
The female animal should be sacrificed as soon as possible after the parturition.  
The neonates should be sacrificed on the next day of birth.
  - (d) Residual viability of the pesticidal microbe in the body  
Count the number of microbe in the organs, tissue and body fluid of the necropsied animals.

(3) Summarization of the results

Based on the results of observation of symptoms, necropsy and number of microbe, a summary should be made as to the following items:  Clinical signs and symptoms  Pregnancy index and gestation period  Fatality and survival during gestation  Effect on the reproductive capability, and presence or absence of abnormal birth  Effect on the body weight of parent animals and offspring  Food consumption  Pathological changes  Abnormality in the shape of neonates  Presence or absence of infection by organ. The pregnancy index consists of copulation rate, pregnancy rate and birth rate, the calculation of which is made as follows:

Copulation rate =

No. of animals which copulated / No. of animals used for mating × 100

Pregnancy rate =

No. of animals pregnant / No. of females which copulated × 100

Birth rate =

No. of female delivering live offspring / No. of pregnant females × 100

(4) Proceeding to the next stage of test

Not particularly required.

**11. Viral carcinogenicity study**

(1) Objective

To evaluate whether a microbial pesticide has any carcinogenicity, this test should be performed in any of the following cases:

- When a virus involved is suspected of having carcinogenicity or is closely related to viruses having carcinogenicity.
- When any infectivity was observed in mammalian cells in the cell culture study.
- In cases where the possibility cannot be denied of the viruses shown in  and  above having got into the microbial pesticide.

(2) Test method

Method should be employed for case by case, according to the carcinogenicity test method for major tumor viruses.

(3) Proceeding to the next stage of test

Not particularly required.

## 12. Immunodeficiency inducing study

(1) Objective

To evaluate whether a microbial pesticide containing a virus as active ingredient has immunodeficiency inducing effect, this study should be performed when the virus involved comes under any of the following:

- When the virus showed any infectivity in the mammalian cells in the cell culture study.
- When the virus is closely related to the viruses known to cause infection or lesion in the immune system of the mammals, thereby inducing a state of immunodeficiency.

Viruses known to induce a state of immunodeficiency in the mammals include the following:

Retroviruses such as cat leukemia virus, mouse AIDS virus, and bovine leukemia virus; and herpesviruses such as human cytomegalovirus.

(2) Test method

Of the above-mentioned immunodeficiency inducing viruses, a special immunodeficiency - inducing study method should be worked out for a virus that is closely related to the virus contained in the microbial pesticide, and a test should be performed accordingly. At the same time, another test should be performed, by the same method, for the virus contained in the microbial pesticide.

Then, it should be proved that the virus contained in the pesticide does not cause such immunodeficiency as brought about by the aforesaid known virus.

(3) Proceeding to the next stage of test

Not particularly required.

## 13. Effect test on primates

(1) Objective

To evaluate whether a microbial pesticide causes any infectivity and pathogenicity in primates, a test should be performed in any of the following cases, by inoculating primates with the pesticidal microbe and by investigating resulting infectivity and pathogenicity.

- Where the pesticidal microbe involved is a virus, and the virus was found to have caused infectivity in any one of the mammalian cells in the cell culture test.

- Where the pesticidal microbe involved is one known to parasitize mammalian cells.
- Where the possibility cannot be denied that a microbial pesticide may contain microbial contaminant(s) known to parasitize mammalian cells.
- Where it is suggested, from the taxonomic viewpoint, that a pesticidal microbe may cause infectivity or pathogenicity in humans.

(2) Test method

As regards necessary type of monkey (Cynomolgus monkey, Rhesus monkey, African green monkey, etc.), number of animals, age, route of inoculation (oral, intravenous, via respiratory tract, via central nervous system, etc.), method of observation, and method of measurement, the matter should be studied for each case separately.

(3) Proceeding to the next stage of test

Not particularly required.

## **V. Cases of hypersensitive reactions that occurred in production and use of microbial pesticides**

### **1. Purpose**

To evaluate whether a microbial pesticide causes hypersensitive reaction in humans, persons who were involved in the production of the microbial pesticide should be observed for the occurrence of any hypersensitive reaction.

### **2. Investigation method**

To examine the occurrence of hypersensitive reaction in humans, persons who were involved in the production or experimental use of a microbial pesticide, an investigation should be conducted by means of monitoring or medical examination.

In addition, a survey of literatures should be made on the cases of hypersensitive reaction that occurred in humans and domestic animals.

### **3. Summarization of the results**

A summary should be made of the following items:

- Technical grade of the active ingredient and end-use product used for exposure, and content thereof
- Date and place of exposure
- Frequency of exposure
- Route of exposure
- Environment and condition under which the exposure test was performed
- Clinical findings
- Other related information

### **4. Proceeding to the next stage of test**

Where it is definitely clear that hypersensitive reaction has occurred, an investigation should be made as to the cause of its occurrence in each case.

## **VI. Results of studies of residual microbial viability on crop plants**

### **1. Purpose**

When a microbial pesticide intended for food crops (including industrial crops and feed crops) was found to have shown effect in the first stage of test concerning safety in humans, a test should be performed, using the said pesticide, to confirm the safety of food crops by investigating its residual viability on food crops.

### **2. Test method**

#### **(1) Test substance**

End-use product

#### **(2) Test crop plants**

As a rule, all food crops should be taken from among the crop plants to be listed in the application for registration.

#### **(3) Preparation of testing samples**

##### **a. Cultivating conditions**

(a) Test plants should be cultivated at two or more cultivating lots, obviously isolated from each other, where variety of crop plant, cultivating method, previous history of the use of agricultural chemicals, weather condition, etc. are clear.

(b) Cultivation should be done by the conventional method.

##### **b. Method of application**

The test substance should be applied evenly according to the method of application to be shown in the application for registration.

For control, non-application area (untreated area) should be set.

##### **c. Time of collecting testing samples**

Testing samples should be collected from crops in a shippable condition.

##### **d. Method of collecting testing samples**

A required quantity of testing samples should be collected evenly from edible portion in each cultivating lot. Injured or damaged portion should not be included in the samples.

##### **e. Preservation of testing samples**

Testing samples should be examined as soon as possible after collection. Where it is difficult to make examination promptly because of unavoidable circumstances such as transportation, the testing samples should be preserved at 5°C or below.

#### **(5) Test items**

Microbial count should be made of the collected testing samples.

### **3. Summarization of the results**

The results of the test should be summarized and recorded.

### **4. Proceeding to the next stage of test**

Not particularly required.

## VII. Ecotoxicology study

### 1. Toxicity study on freshwater fish

#### (1) Purpose

To evaluate the effects of a microbial pesticide on freshwater fish, the test freshwater fish should be exposed in water to the pesticidal microbe in a high concentration, and thereby the effects of the microbial pesticide on the test freshwater fish should be examined.

However, when there is no possibility of exposure, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

#### (2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test animals: As a rule, carp (*Cyprinus carpio*) or rainbow trout (*Oncorhynchus mykiss*) should be used (yearling fish; about 5 g; in body weight, after acclimatization for 1 week or more).

c. Test groups to be used

Control group: Untreated group

Treated group: The use level per unit area should be 1,000 as strong in concentration as the level for a water tank 15 cm in depth used by dropping the pesticide directly into the water.

When any effect has been observed at the above concentration, a concentration-response test should be performed to clarify the level at which an effect is seen.

The number of fish used should be 10 or more per test group, and the test should be made three times per test group.

d. Exposing method and raising conditions

The test fish should be exposed for 30 days in the test solution, with a fixed concentration, of the test substance, under semi-static system. The water for acclimation should be dechlorinated tap water or sterilized underground water of good quality, with pH at 6.5 - 8.0. The volume of water for the test should be 1L or more/g in body weight. The temperature of water should be the optimum temperature for the type of fish used plus/minus 2°C. The dissolved oxygen concentration should be at least 60% of the saturation concentration. As regards feed, a prearranged quantity (about 3%, on dry basis, of the weight of fish) of formula feed should be given every day.

e. Test period

As a rule, for 30 days from the starting day of exposure. When any effect has been observed in the course of test, the test period should be extended until recovery, death or a moribund state can be confirmed.

f. Test items

(a) Test of water quality

Water temperature, dissolved oxygen concentration, pH, total hardness and microbial concentration in the test aquaria should be measured at regular intervals.

(b) Measurement of body weight

The fish should be weighed at the start of the test and at the time of necropsy.

(c) Observation of signs and symptoms  
Observation should be made every day as to appearance, feed eating situation, abnormal swimming, death, etc. A fish is considered dead when it does not respond to a stimulus given.

(d) Pathological examination  
When a fish dies in the course of the test, it should be necropsied promptly, and the surviving fishes should all be sacrificed for necropsy, at the end of the test. The date of death and findings from the necropsy should be recorded, including the presence of any infection caused by the pesticidal microbe.

(3) Summarization of the results

The results should be summarized for each of the test items. When any effect has been observed, the maximum no-effect concentration should be determined.

(4) Proceeding to the next stage of test

- a. When no effects have been observed in any of the test fish, no further tests are required.
- b. When any effect has been found in test fish, a study of the kinetics of the pesticidal microbe in the environment should be performed.

## 2. Toxicity study on freshwater invertebrates

(1) Purpose

To evaluate the effects of a microbial pesticide on freshwater invertebrates, the test animals should be exposed, in water, to the pesticidal microbe in a high concentration, and thereby the effects of the pesticidal microbe on the test invertebrate freshwater animals should be examined.

When there is no possibility of exposure of invertebrate freshwater animals to the pesticidal microbe, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

- a. Test substance: Technical grade of the active ingredient
- b. Test animals

Any one of water flea species *Daphnia pulex*, *Daphnia carinata*, and *Daphnia magna*. (Animals should be those whose growth conditions are clear, and which are within 24 hours after birth.)

- c. Test groups to be set

Control group: Untreated group

Treated group: The use level per unit area should be 1,000 times as strong in concentration as the level for a water tank 15 cm in depth used by dropping the pesticidal microbe directly into the water tank.

When any effect has been observed at the above concentration, a concentration-response test should be performed to clarify the concentration at which an effect is seen.

The number of water fleas used should be 20 or more per test group, and the test should be performed three times per test group.

- d. Exposing method and growing method

The test animals should be exposed for 21 days in the test solution, with a fixed concentration, of the test substance, under semi-static system. The water for culturing should be dechlorinated tap water or sterilized underground water of good quality, with pH at 6.5 - 8.0. The volume of water for the test should be 40 ml or more/animal. The water temperature should be  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .

The dissolved oxygen concentration should be at least 60% of the saturation concentration. A prearranged quantity of feed (algae) should be given.

e. Test period

As a rule, for 21 days from the starting day of exposure. When any effect has been observed in the course of the test, the test period should be extended until recovery, death or a moribund state can be confirmed.

f. Test items

(a) Test of water quality

Water temperature, dissolved oxygen concentration, pH, total hardness, and microbial concentration in the test vessels should be measured at regular intervals.

(b) Observation of symptoms

The test animals should be observed every day for appearance, abnormal swimming, death, etc. An animal is considered dead when its antennae have ceased to move.

(c) Observation of reproductive capability

Newly born young animals and eggs should be counted every other day. These should be removed after counting.

(3) Summarization of the results

A summary should be made according to (3), 1. of Section VII.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

### 3. Avian toxicity study

(1)

To evaluate the effects of a microbial pesticide on birds, the pesticidal microbe in a high concentration should be given orally to birds, and thereby its effects on test birds should be examined.

However, when there is no possibility of the birds being exposed to the pesticidal microbe, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test animals:

Quails or mallard ducks (14 - 28 days of age, body weight within average weight  $\pm 20\%$ , after acclimatization for one week)

c. Test groups to be set

Control group:

Solvent-treated group

Untreated group (This group is needed where the effect of the solvent is unknown.)

Treated group:

$10^8$  unit/0.2 ml/bird should be given orally for 5 days.

If above dose level has shown any pathogenicity and toxicity, a dose-response test should be performed to clarify the dose level at which pathogenicity and toxicity are seen.

The number of test birds should be at least 10 per test group, and test should be performed three times per test group

d. Test period

As a rule, for 30 days from the start of administration. When any pathogenicity and toxicity have been observed in the course of the test, the test period should be extended until recovery, death or a state of moribund can be confirmed.

e. Raising conditions

A suitable quantity of feed, not containing antibiotic, for newly-hatched birds should be given according to the growth of young birds. Drinking water should be given ad libitum, with water changed every day. Temperature and humidity should be kept at optimum levels according to the age of birds. As regards lighting, a cycle of "16-hour lighting followed by 8-hour dark period" should be employed.

f. Observation items

(a) Observation of signs and symptoms

Birds should be observed every day for such symptoms as ruffled up feathers, drooping wings, lost strength, dangling head, closed eyes, salivation, diarrhea, dyspnea, weakness and death.

(b) Measurement of body weight

The birds should be weighed just before administration, and 7, 14, 21 and 28 days after the start of administration, and at the time of death.

When the test period has been extended, the measurement should be made every week.

(c) Pathological examination

When a bird has died in the course of the test, it should be necropsied promptly, and the surviving birds should all be sacrificed for necropsy, at the end of the test. The date of death and findings from necropsy should be recorded, including the presence of infection, by organ, caused by the pesticidal microbe.

(3) Summarization of the results

Based on the results of observation and pathological examination, a summary should be made of the following items:

General signs and symptoms  Death rate  Changes in body weight  Pathological changes  Presence or absence of infection by organ.

When any pathogenicity and toxicity have been observed, the maximum no-effect level should be determined.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

#### 4. Study of effects on non-target plants

(1) Purpose

To evaluate the effects of a microbial pesticide on non-target plants, the non-target plants including crop plants should be once exposed to the pesticidal microbe in a high concentration, and thereby the effects on the test plants should be examined.

However, when there is no possibility the plants being exposed to the microbial pesticide, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test plant

(a) As a rule, at least 4 families and 6 species of dicotyledonous plants and at least 2 families and 4 species of monocotyledonous plants should be selected from among economically important plants.

(b) In tests of microbial pesticides for destroying weeds and pesticides closely related to plant-pathogenic microbes, at least 2 species of economically important plants or plants useful for the maintenance of ecological system should be selected, in addition to the above-mentioned plants, from among the plants closely related to the target plants and from the plants sensitive to plant-pathogenic microbes closely related to the pesticidal microbe.

(c) In tests of microbial pesticides used in water, an additional test should be performed using algae, based on OECD Test Guidelines 201 (Alga, growth inhibition test).

c. Test groups to be set

Control group:

Untreated group ( When a wetting agent is used in the treated group, it should also be used in the untreated group.)

Reference group (In the case of a microbial pesticide for destroying weeds, the microbe contained in the said pesticide should be applied to the target plants, and this should be used as control group. In the case of a microbial pesticide closely related to plant-pathogenic microbes, a plant-pathogenic microbe taxonomically closely related to the microbial pesticide should be applied to susceptible plants, and this should be used as control group.)

Treated group:

The concentration used should 10 times the maximum concentration to be shown in the application for registration.

When any effect has been observed at the above concentration, a dose-response test should be performed to clarify the concentration at which an effect is seen.

The test should be performed three times per test group.

d. Exposing method

Judging from the type of pesticidal microbe, its mechanism of action and type of test plant, the exposure route and growth stage where the test plant is most likely to be exposed to the microbe should be selected. To make the pesticidal microbe easily stickable to the plant, a suitable quantity of a wetting agent may be added to the suspension of the pesticidal microbe.

e. As a rule, Test period should be for 3 weeks after administration

f. Cultivating conditions

To maintain healthy growth of the plant, care should be taken about appropriate fertilizer application, water, light, temperature and humidity. Other agricultural chemicals should not be used.

g. Observation items

(a) Observation of signs and symptoms

The plant should be observed, at regular intervals, to examine the growth, and development of any disease, mold, etc.

(b) Pathological examination

When a plant has withered in the course of the test, its roots, leaves, vessels, etc. should be examined to see if there is any infection by the pesticidal microbe.

(3) Summarization of the results

A summary should be made according to (3), 1. of Section VII.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

**5. Study of effects on non-target insects**

(1) Purpose

To evaluate the effects of a microbial pesticide on non-target insects, parasitic insects or predatory insects should be once exposed to the pesticidal microbe in a high concentration, and thereby the effects on the test insects should be examined.

However, when there is no possibility of the non-target insects being exposed to the microbial pesticide, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test insects

Three species belonging to at least two of the seven orders listed below should be selected:

Parasitic Diptera (parasites such as takinid flies)

Parasitic Hymenoptera (parasites such as trichogramma spp.)

Predatory Hemiptera (mirid bug etc.)

Predatory Coleoptera (lady beetle etc.)

Predatory Neuroptera (lacewing etc.)

Predatory Acarina (predatory mites)

Predatory Arachnida (*Lycosa* spp. etc.)

c. Test groups to be set

Control group:

Untreated group (When a wetting agent is used in the treated group, it should also be used in the untreated group.)

Treated group:

The concentration used should be 10 times the maximum concentration to be shown in the application for registration of the microbial pesticide concerned.

When any effect has been observed at the above concentration, a dose-response test should be performed to clarify the concentration at which an effect is seen.

The test should be performed three times per test group.

d. Exposing method

Judging from the type of pesticidal microbe, its mechanism of action and type of test insect, the exposure route in which the test insect is most likely to be exposed to the pesticidal microbe should be selected. To make the pesticidal microbe easily stickable to the insects, a wetting agent may be added to the suspension of the pesticidal microbe.

e. Test period

A suitable period should be set according to the type of pesticidal microbe, type of test insect, etc.

f. Observation items

(a) Observation of signs and symptoms

Although observation items differ with type of test insect, pathogenetic situation and test method, such items as number of days required to become pupa, pupation rate, oviposition rate, hatching rate (fertilization rate), and survival or death should be observed at regular intervals.

(b) Pathological examination

When, in the course of the test, an insect has died or any effect has been observed, the presence of any infection in it caused by the pesticidal microbe should be examined.

(3) Summarization of the results

A summary should be made according to (3), 1. of Section VII.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

## 6. Study of effects on honey bees

(1) Purpose

To evaluate the effects of a microbial pesticide on honey bees, honey bees should be exposed to the pesticidal microbe in a high concentration, and thereby the effects on the test honey bees should be examined.

However, when there is no possibility of the honey bees being exposed to the pesticidal microbe, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test honey bees

Honey bees (Imagoes of the same age, 3 - 7 days after emergence)

c. Test groups to be set

Control group: Untreated group

Treated group:

The concentration used should be 10 - 100 times the maximum concentration to be shown in the application for registration of the pesticidal microbe concerned, and a highest possible concentration should be used.

When any effect has been observed at the above concentration, a dose-response test should be performed to clarify the concentration at which an effect is seen.

The number of bees used should be 25 per test group, and the test should be performed three times per test group.

d. Exposing method and raising method

Where the pesticidal microbe involved is a filamentous fungus, the pesticidal microbe should be sprayed to the bees, using a sprayer, until they are completely covered with the pesticidal microbe. When the microbe does not stick well to the bees, a wetting agent not harmful to the bees should be used.

Where the pesticidal microbe involved is one other than filamentous fungus, a sucrose solution (20 - 50%, it should be confirmed that the solution has no effect on the microbe) mixed with the pesticidal microbe should be left in a feeder for 48 hours for the bees to eat it.

After spraying (after the body surface with liquid has dried) or after giving the above sucrose solution, the bees should be given feed not containing the pesticidal microbe. The feed and water should be renewed from time to time.

e. Test period

As a rule, Test period should be for 20 days after administration

f. Observation items

(a) Observation of signs and symptoms

The first observation as to bees' behavior, death, etc. should be made 4 hours after spraying. Thereafter, observation should be made accordingly every day. As regards any abnormal behavior, judgment should be made by comparison between the treated group and the control group.

(b) Pathological examination

When a bee has died in the course of the test, it should be examined without delay to avoid secondary infection, as to any infection in it caused by the pesticidal microbe.

When collecting dead bee(s), care should be taken not to incite bees to bring trouble.

(3) Summarization of the results

A summary should be made according to (3), 1. of Section VII.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

## **7. Study of effects on silkworms**

(1) Purpose

To evaluate the effects of a microbial pesticide on silkworms, silkworms should be exposed to the pesticidal microbe in a high concentration, and thereby the effects on test silkworms should be examined.

However, when there is no possibility of mulberry trees being exposed to the pesticidal microbe, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test silkworms

4th instar larvae of silkworm just after moulting

c. Test groups to be set

Control group:

Untreated group (When a wetting agent is used in the treated group, it should also be used in the untreated group.)

Treated group:

The concentration used should be 10 times the maximum concentration to be shown in the application for registration of the microbial pesticide concerned.

When any effect has been observed at the above concentration, a dose-response test should be performed to clarify the concentration at which an effect is seen.

The number of test silkworms used should be 50 per test group, and the test should be performed twice per test group.

d. Exposing method and raising conditions

Control group:

Mulberry leaves not contaminated with the pesticidal microbe, or artificial diet (not containing antibiotic) should be given to the silkworms every day.

Treated group:

Where the pesticidal microbe involved is a filamentous fungus, the silkworms should be immersed in its suspension. After that, the silkworms should be given mulberry leaves every day that are not contaminated with the pesticidal microbe. To make the microbe easily stickable to the silkworms, a wetting agent may be added to the suspension.

Where the microbe involved is one other than filamentous fungus, mulberry leaves immersed in a suspension of the pesticidal microbe and then dried, or artificial diet mixed with the said suspension (0.05 - 0.1 ml/g) should be given to the silkworms for 24 hours. After that, the silkworms should be given untreated mulberry leaves or artificial diet every day. The artificial diet to be selected should be one that the silkworms like to eat and is good for their growth.

e. Test period

As a rule, for 20 days after exposure As a rule, Test period should be for 20 days after administration

f. Observation items

(a) Observation of signs and symptoms

The number of dead silkworms should be checked every day, and when necessary, such items as days of 4th and 5th instar, number of cocoon-spinning silkworms, pupation rate, weight of cocoon, weight of cocoon layers and symptom of poisoning should also be observed.

(b) Pathological examination

When a silkworm has died in the course of the test, it should be examined as to the presence of any infection etc. caused by the pesticidal microbe.

(3) Summarization of the results

A summary should be made according to (3), 1. of Section VII.

(4) Proceeding to the next stage of test

Procedures should be taken according to (4), 1. of Section VII.

## 8. Study of effects on soil microbes

(1) Purpose

To evaluate the effects of a microbial pesticide on soil microbes (bacteria, actinomycetes and fungi), the pesticidal microbe in a high concentration should be mixed with the soil, and thereby the effects on the soil microbes should be examined.

However, when there is no possibility of the soil being exposed to the pesticidal microbe, because of scientific grounds from the viewpoint of biological properties of the microbe or judging from the method of use, this test may be omitted.

Where considered necessary, the effects of the pesticidal microbe on the carbon metabolism and nitrogen metabolism of the soil microbes should also be investigated.

(2) Test method

a. Test substance: Technical grade of the active ingredient

b. Test soil

When the microbial pesticide is one intended for use in paddy field, paddy field soil should be used, and when the microbial pesticide is one intended for use in upland field, upland soil should be used. The area used should be about 1 m × 1 m for both, and the areas should be isolated from each other, and should be those under proper maintenance.

c. Test groups to be set

Control group: Untreated group

Treated group:

A quantity 10 times the normal use level per unit area should be mixed with the soil, and the depth of mixing should be 20 cm.

When any effect has been observed at the above use level, a dose-response test should be performed to clarify the use level at which an effect is seen.

The test should be performed three time per test group.

d. Test period

As a rule, Test period should be for 3 months after administration

e. Collection of soil samples

Sample should be taken from at least 4 spots per test area, using a soil collecting tube (approximate size: 4 cm in diameter × 4 cm in length), and the samples thus taken should be well mixed. As a rule, samples should be taken 1, 10, 30 and 90 days after the start of the test.

f. Observation items

Microbial count should be made of each of the bacteria, actinomycetes and fungi in the soil sample. The counting method should be one high in selectivity, sensitivity and reliability according to the type of microorganism.

(3) Summarization of the results

A summary should be made for each of the observation items. When any effect has been observed, the maximum no-effect level should be determined.

- (4) Proceeding to the next stage of test  
Procedures should be taken according to (4), 1. of Section VII.

## **VIII. Study on the behavior in the environment**

### **1. Purpose**

When any adverse effect by microbial pesticide has been observed on the organisms in the environment, the behavior of the microbial pesticide, such as the survival ability and the propagation ability in the environment, should be investigated in order to evaluate the possibility of exposure to the said species of the organisms.

### **2. Test method**

Taking sufficiently into consideration such factors as biological properties of the pesticidal microbe, method of using the microbial pesticide, the species of the organisms on which any adverse effect was observed in the studies of effects on the organisms in the environment, and the biological characteristics of the affected organisms, a suitable test method should be selected for each case separately so that the possibility of the said species of the organisms being exposed to the said microbial pesticide in the environment can be evaluated adequately.

### **3. Proceeding to the next stage of test**

- (1) When no possibility was observed of the said species of the organisms being exposed to the pesticidal microbe, no further tests are required.
- (2) When any possibility was observed of the said species of the organisms being exposed to the pesticidal microbe, further studies should be duly considered.