F.No. 26-01/2021-CIR-I
भारत सरकार
Government of India
कृषि एवं किसान कल्याण मंत्रालय
Ministry of Agriculture & Farmers Welfare
कृषि, सहकारिता एवं किसान कल्याण विभाग
Department of Agriculture, Co-operation & Farmers Welfare
वनस्पति संरक्षण, संगरोध एवं संग्रह निदेशालय
DIRECTORATE OF PLANT PROTECTION, QUARANTINE & STORAGE
केंद्रीय कीटनाशी बोर्ड ए वम पंजीकरण समिति
Central Insecticides Board and Registration Committee
एन. एच. 4, फरीदाबाद (हरियाणा)-121001
N.H. IV, FARIDABAD (HARYANA)-121001
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Dated: 07 January, 2021

PUBLIC NOTICE

Subject-Harmonization of existing toxicological guidelines/protocols for registration of bio-pesticides in India-regarding.

The matter was placed before the RC at agenda item No. 10.16 in 422nd meeting held on 23.10.2020 and RC has approved the guidelines/protocols drafted by the sub-committee and decided that a public notice may be displayed on the website for seeking comments of the stakeholders.

In view of the above, stakeholders are requested to submit their comments within 30 days period from the date of uploading of the Public Notice on the website of Dte. of PPQ&S. The reply may be sent through email to cibsecy@nic.in, sarita.bhalla@nic.in and socir2.ppqs-agri@gov.in.

This has the approval of Secretary (CIB&RC).

07/01/21

(J.K.Meena)
Section Officer

Copy to :
1. Pesticide Associations
2. Chairman, Registration Committee
3. PPS to JS (PP)
4. PPS to PPA
5. PPS to Secretary (CIB&RC)
6. IT Cell, HQ, Faridabad for uploading the same on the website.

Acute Dermal (Toxicity/Infectivity/Pathogenicity) Study

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide.

Purpose: The purpose of the acute dermal study is to provide initial information on the toxicity, infectivity, and/or pathogenicity of microbial pesticides using a single dose exposure for prescribed observation period. It provides information on health hazards likely to arise from a single exposure by the dermal route in small laboratory animals.

Principle/Rationale: The microbial pesticide to be tested is applied in a single high dose to the skin of experimental animals. Subsequently, observations of effects and deaths are made. Animals that die during the test are necropsied and at the end of the test the surviving animals are humanely sacrificed (using approved methods) and necropsied. Infectivity of the microbial pesticides is evaluated periodically during and at the end of the test.

Test animals

Species: Although several mammalian test species may be used, the albino rabbit is the preferred species. Commonly used laboratory strains should be employed. If another species is used, the investigator should provide justification/ reasoning for the alternative selection. All test animals should be free of parasites or pathogens. Females should be nulliparous and non pregnant. All experiments must be conducted as per Good Laboratory Practices (GLP) and CPCSEA guidelines.

Age: Young adult animals should be used.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Gender: Equal numbers of animals of each gender (sex) are required.

Housing and Feeding Conditions

Temperature: 22°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: Ad libitum standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days prior to dosing by keeping them in individual cages.

Groups and numbers of animals required

Test group

At least 10 animals (5 animals of each gender) should be used. Females should be nulliparous and non pregnant.

Control group

Neither a concurrent untreated nor vehicle control group are required except when the toxicity of the vehicle is unknown.

Dosing (Dose level)

A single dose level of at least 10^9 Colony Forming Units (CFU) or 2 gm/ kg body weight of the Microbial Pesticides per test animal should be used.

Administration method

Preparation of animal skin

i) Approximately 24 h before the test, fur should be removed from the dorsal and ventral area of the trunk of each test animal by clipping or shaving.

ii) Not less than 10 percent of the body surface area should be cleared for application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

Application of the test substance

i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area.

ii) The test substance should be held in contact with the skin with porous gauze and a nonirritating tape throughout a 24–h exposure period. The test site further should be covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended.

iii) At the end of the exposure period, residual test substance should be removed, where practical, using water.

Observation

i) A careful clinical examination should be made at least once each day.

ii) Cage side observations should include, but not be limited to changes in skin including signs of irritation and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, behavior pattern, particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep, and coma.

iii) Weight of the individual test animals should be recorded, shortly prior to the administration of test substance, weekly (at least) after the dosing and at the time of death or interim or final sacrifice. All surviving animals are to be weighed before they are humanely sacrificed.

iv) The time of death should be recorded as precisely as possible.

v) At the end of the 24–h exposure period, and daily thereafter, any signs of skin irritation should be recorded and scored.

Gross pathology

Consideration should be given to performing a gross necropsy of all animals if indicated by the appearance of toxic effects. If done, all gross pathological changes should be recorded.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity and mortality as the study end points.

Reference: OPPTS 885.3100; Acute Dermal toxicity/pathogenicity

Acute Inhalation (Toxicity/Infectivity/Pathogenicity) Study

Objective: To assess and evaluate the toxic orpathogenic characteristics of a Microbial Pesticide.

Purpose: The purpose of the acute inhalationtoxicity study is to assess and evaluatethe toxic characteristics of microbial pesticides that may be inhaled such as a gas, volatile substance, or aerosol/particle. It provides information on health hazards likely to arise from short-term exposure by the inhalation route.

Principle/Rationale: An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.Several groups of experimental animals are exposed to the test substance in graduated concentrations for a defined period, one concentration being used per group. Animals that die during the test are necropsied, and at the end of the test the surviving animals are sacrificed and necropsied ethically. Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out. Infectivity of the Microbial Pesticides is evaluated periodically during the test, and at the end of the test.

Test animals

Species: Rat is the recommended rodent species. Commonly used laboratory strains should be selected. If another species is used, justification/reasoning for the alternative selection should be provided. All test animals should be free of parasites or pathogens and experiments must be conducted as per Good Laboratory Practices (GLP) and CPCSEA guidelines.

Age: Between 8 and 12 weeks at the time of dosing.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Gender: Equal numbers of animals of each gender (sex) are required.

Housing and Feeding Conditions
Temperature: 22°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).

Lighting: 12 hours light and dark cycle
Diet and water:*Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization:The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Groups and numbers of animals required

Several groups of experimental animals are exposed to the test substance in graduated concentrations for a defined period, one concentration being used per group. At least five animals are used at each concentration and they should be of one sex. After completion of the study in one sex, at least one group of five animals of the other sex is exposed to establish that animals of this sex are not markedly more sensitive to the test substance.

Test group

At least 10 animals (5 animals of each gender) should be used. Females should be nulliparous and non pregnant. A sufficient number of additional animals should be taken for interim sacrifice to determine infectivity.

Control group

i) A concurrent "untreated control" group of four animals per gender is required. Half of the animals in the control group (i.e., two animals per gender) should be housed separately from the test group of animals dosed with Microbial Pesticides. The remainder of control group [shelf control] animals (comprising of two males&females) should be housed with the dosed/treated animals to evaluate infectivity of test compound.

ii) A separate "vehicle control" group is not required except in situations where the toxicity of the vehicle is not known.

iii) An additional control group including two males&two femalesare dosed to prove/evaluate the toxic propertiesof inactivated (i.e., rendered incapable of reproduction or germination) microbial pesticides. Inactivation should be done by means/methods that allows the maintenance of structural integrity of the microbial pesticides.

Dosing (Dose level)

Several groups of experimental animals are exposed to the Microbial Pesticides test substance in graduated concentrations for a defined period, one concentration being used per group.

Vehicle

The recommended vehicle for the pesticides is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the Microbial pesticides will be distributed, mixed, suspended, or diluted for application. A vehicle control group should be used when historical data are not available or adequate to determine the acute inhalation toxicity of the vehicle or when a vehicle other than water is used to help generate an appropriate concentration of the substance in the atmosphere

Equipment

1) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of at least 10 air changes per hour, an adequate oxygen content of at least 19 percent, and uniform conditions throughout the exposure chamber. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas. It is normally not necessary to measure chamber oxygen concentration if airflow is adequate.

2) The selection of a dynamic inhalation chamber should be appropriate for the test article and test system. Where a whole body chamber is used to expose animals to an aerosol, individual housing must be used to minimize crowding of the test animals and maximize their exposure to the test substance. To ensure stability of a chamber atmosphere, the total volume of the test animals should not exceed 5 percent of the volume of the test chamber. It is recommended, but not required, that nose-only or head-only exposure be used for aerosol studies in order to minimize oral exposures due to animals licking compound off their fur. The animals should be acclimated and heat stress minimized.

Physical measurements

Measurements or monitoring should be made of the following:

1) The rate of air flow should be monitored continuously, but recorded at least 3 times during the exposure.

2) The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable and monitored continuously or intermittently depending on the method of analysis. Chamber concentration may be measured using gravimetric or analytical methods as appropriate. If trial run measurements are reasonably consistent (±10 percent for liquid aerosol, gas, or vapor; ±20 percent for dry aerosol), then two measurements should be sufficient. If measurements are not consistent, three to four measurements should be taken. Whenever the test article is a formulation, the analytical concentration must be reported for the total formulation, and not just for the active ingredient (AI). It is not necessary to analyze inert ingredients provided the mixture at the animal's breathing zone is analogous to the formulation; the grounds for this conclusion must be provided in the study report. If there is some difficulty in measuring chamber analytical concentration due to precipitation, nonhomogeneous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary.

3) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. The Mass median aerodynamic diameter (MMAD) particle size range should be between 1–4 μm. The particle size of hygroscopic materials should be small enough when dry to assure that the size of the swollen particle will still be within the 1– 4 μm range. Measurements of aerodynamic particle size in the animal's breathing zone should be measured during a trial run. If MMAD values for each exposure level are within 10 percent of each other, then two measurements during the exposures should be

sufficient. If pretest measurements are not within 10 percent of each other, three to four measurements should be taken.

4)Temperature and humidity should be monitored continuously and recorded at least 3 times during exposure. The temperature at which the test is performed should be maintained at 22 ± 2 °C. The relative humidity should be maintained between 30 and 70 percent humidity, but in certain instances (tests of aerosols) this may not be practicable.

Exposure duration and levels

i) Shortly before exposure, the animals are weighed and then exposed to the test concentration in the designated apparatus for 4 h after equilibration of the chamber concentrations. Other durations may be needed to meet specific requirements. Food should be withheld during exposure. Water may also be withheld in certain circumstances.

ii) Three concentration levels should be used and spaced appropriately to produce a concentration-response curve and permit an estimation of the median lethal concentration. Range-finding studies using single animals may help to estimate the positioning of the test groups so that no more than three concentration levels will be necessary.

Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset, and length of recovery period, and thus may be extended when considered necessary. The time at which signs of toxicity appear, their duration, and the time 5 of death are important, especially if there is a tendency for deaths to be delayed.

Observation of animals

1)A careful clinical examination should be made at least once each day.

2) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals.

3)Observations should be detailed and carefully recorded, preferably using explicitly defined scales. Observations should include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior (e.g., self mutilation, walking backwards).

4) Individual weights of animals should be determined prior to exposure, weekly after exposure, and at death. Changes in weights should be calculated and recorded when survival exceeds 1 day.

5) The time of death should be recorded as precisely as possible.

Gross pathology

1)At the end of the test, surviving animals should be weighed and sacrificed.

2) A gross necropsy should be performed on all animals under test, with particular reference to any changes in the respiratory tract. All gross pathology changes should be recorded.

3) If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis. Necropsies should be performed as soon as possible, normally within a day or two.

Additional evaluations

In animals surviving 24 h or more, microscopic examination of organs showing evidence of gross pathology should be considered since it may yield useful information.

Result assessment

The LC50 value should be considered in conjunction with the observed toxic effects and the necropsy findings. The LC50 value is a relatively coarse measurement useful only for classification and labeling purposes and an expression of the lethal potential of the test substance following inhalation. Reference should always be made to the experimental animal species and exposure duration in which the LC50 value was obtained. An evaluation should include the relationship, if any, between exposure of animals to the test substance and the incidence and severity of all abnormalities including behavioral and clinical abnormalities, gross lesions, body weight changes, mortality, and other toxic effects. The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity and mortality as the study end points.

Reference: OPPTS 870.1300; Acute inhalation toxicity

Acute Intraperitoneal/Intravenous (Toxicity/Infectivity/Pathogenicity) Study

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide.

Purpose: The purpose of the acute intraperitoneal/intravenous study is to provide initial information on human, animal and environmental health hazards likely to arise based on the toxicity, infectivity, and/or pathogenicity of Microbial Pesticides using a single dose exposure for prescribed observation period.

Principle/Rationale: The test Microbial Pesticide is administered by intravenous/intraperitoneal injection in a single dose to experimental animals. Subsequent observations of effects and deaths are made and rate of clearance of the Microbial Pesticides is estimated. Animals that die during the test are necropsied and at the end of the test the surviving animals are humanely sacrificed (using approved methods) and necropsied. Infectivity of the microbial pesticides is evaluated periodically during and at the end of the test.

Test animals

Species: Rat or mouse is the recommended rodent species. Commonly used laboratory strains should be selected. If another species is used, justification/reasoning for the alternative selection should be provided. All test animals should be free of parasites or pathogens and experiments must be conducted as per Good Laboratory Practices (GLP) and CPCSEA guidelines.

Age: Between 8 and 12 weeks at the time of dosing.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Gender: Equal numbers of animals of each gender (sex) are required.

Housing and Feeding Conditions

Temperature: 22°± 3°C

Relative Humidity: 50-60% (Relative humidity should never be below 30% or above 70% at any given point of time).

Lighting: 12 hours light and dark cycle

Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).

Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days prior to dosing by keeping them in individual cages.

Groups and numbers of animals required

Test group
At least six animals (3 animals of each gender) should be used. Females should be nulliparous/non pregnant. A sufficient number of additional animals should be taken for interim sacrifice to determine infectivity.

Control group

i) A concurrent "untreated control" group of four animals per gender is required. Half of the animals in the control group (i.e. two animals per gender) should be housed sepaprately from the test group of animals dosed with microbial pesticides. The remainder of control group (shelf control) animals (comprising of two males & females) should be housed with the dosed/treated animals to evaluate infectivity of test compound.

ii) A separate "vehicle control" group is not required except in situations where the toxicity of the vehicle is not known.

iii) An additional control group including two males &two females are dosed to prove/ evaluate the toxic properties of inactivated (i.e., rendered incapable of reproduction or germination) microbial pesticides. Inactivation should be done by means/methods that allows the maintenance of structural integrity of the microbial pesticides.

Dosing (Dose level)

A single dose level of at least 10^9 Colony Forming Units (CFU) or 2 gm/ kg body weight of the Microbial Pesticides per test animal should be used.

Vehicle

The recommended vehicle for the pesticides is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the Microbial pesticides will be distributed, mixed, suspended, or diluted for application.

Administration method

Volume

The maximum volume of liquid that can be administered via intravenous or intraperitoneal routes at one time depends on the size of the test animal. Variability in test volume should be minimized.

Dose quantification

Techniques used to quantify the units of microbial pesticides in any dose will depend on the group of microorganisms to which the Microbial pesticides belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. Quantification should be done concurrently with testing.

Exposure

The test substance should be administered via appropriate needle and syringe in a single dose. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 h.

Observation

After dosing the animal (dosed/control) should be observed for a period of at least 21 days. However, the duration of observation should be determined by the type of microbial pesticide administered and its rate of clearance from the test animals. Duration of the observation period also would depend on the time at which signs of toxicity and pathology appear and disappear, and the time of death of the animals. The following parameters should be observed:

i) Observations should be made on individual animals at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), thereafter at least once daily during the whole observation period.

ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous systems, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

iii) The rectal temperature of each animal will be recorded in the morning and evening, daily, throughout the observation period.

iv) Weight of the individual test animals should be recorded, shortly prior to the administration of test substance, weekly (at least) after the dosing and at the time of death or interim or final sacrifice. All surviving animals are to be weighed before they are humanely sacrificed.

v) Animals found in a moribund condition or showing signs of severe distress will be humanely sacrificed.

vi) All test animals should be subjected to gross necropsy at the time of death or interim or final sacrifice to record gross pathological findings. If any sign and /or symptom is suggestive, histopathology, biochemical and haematological parameters may be done.

Gross pathology

A gross necropsy of all animals should be performed. All gross pathological changes should be recorded.

Rate of Clearance of test substance (microbial pesticide)

Intravenous injection only. The test animals sacrificed should be examined for the presence of the microbial pesticides to estimate clearance after administration. Blood from test animals should be collected during the study and examined for the presence of the Microbial pesticides to estimate clearance of the Microbial pesticides after dosing. The first analysis

should be done on three animals per sex as soon as reasonably possible after dosing. The Recovery values, detection and sensitivity limits of test substance (microbial pesticide) should be done using appropriately sensitive analytic method.

Infectivity evaluation

Intravenous injection only. For infectivity and persistence determinations, the microbial pesticide should be enumerated by using sensitive techniques for its presence in tissues, organs, kidneys, brain, liver, lung, spleen, blood, representative lymph nodes, caecum contents, and, where appropriate, from lesions and the inoculation site. (ii) Other tissues, organs, and body fluids may have to be examined as indicated by the nature of any toxic and pathogenic effects observed. Homogenized samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces need to be cultured on selective medium to determine the infectivity/Pathogenicity.

Interim sacrifice

For evaluating infectivity and clearance, the microbial pesticides should be enumerated from tissues, organs, and body fluids of three treated animals per gender, sacrificed at 3 days after, and at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to establish a pattern of clearance adequately. The microbial pesticide isolated from the tissues, organs, and body fluids of the *''shelf control''* group at final sacrifice should be enumerated.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity, rate of clearance and mortality as the study end points.

Reference: OPPTS 885.3200; Acute injection toxicity/pathogenicity

Acute Oral (Toxicity/Infectivity/Pathogenicity) Study

Objective: To assess and evaluate the toxic or infective and pathogenic characteristics of a microbial pesticide.

Purpose: The purpose of the acute oral toxicity study is to provide initial information on the toxicity, infectivity, and/or pathogenicity of microbial pesticides using a single dose exposure for prescribed observation period. It provides information on health hazards likely to arise from a single exposure by the oral route in small laboratory animals.

Principle/Rationale: The test microbial pesticide is administered orally by gavage in a single dose to experimental animals. Subsequent observations of effects, deaths and rate of clearance of the microbial pesticides is observed/estimated. Animals that die during the test are necropsied and at the end of the test the surviving animals are humanely sacrificed (using approved methods) and necropsied. Infectivity of the microbial pesticides is evaluated periodically during and at the end of the test.

Test animals

Species: Rat and mouse are the recommended rodent species. Commonly used laboratory strains should be selected. If another species is used, justification/reasoning should be provided. All test animals should be free of parasites or pathogens. All experiments must be conducted as per Good Laboratory Practices (GLP) and CPCSEA guidelines.

Age: Between 8 and 12 weeks at the time of dosing.

Weight: Weight variation should be within ± 20 % of the mean ideal weight of the animal.

Gender: Equal numbers of animals of each gender (sex) are required.

Housing and Feeding Conditions

Temperature: 22° ± 3°C
Relative Humidity: 50-60% (Relative humidity should never be below 30% or above 70% at any given point of time).
Lighting: 12 hours light and dark cycle.
Diet and water: *Ad libitum* standard laboratory diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days prior to dosing by keeping them in individual cages.

Groups and numbers of animals required

Test group

At least six animals (three animals of each gender) should be used. Females should be nulliparous/non-pregnant. A sufficient number of additional animals should be taken for interim sacrifice to determine infectivity.

Control group

i) A concurrent "untreated control" group of four animals per gender is required. Half of the animals in the control group (i.e., two animals per gender) should be housed separately from the test group of animals dosed with Microbial Pesticides. The remainder of control group [shelf control] animals (comprising of two males & females) should be housed with the dosed/treated animals to evaluate infectivity of test compound.

ii) A separate "vehicle control" group is not required except in situations where the toxicity of the vehicle is not known.

iii) An additional control group including two males & two females are dosed to prove/evaluate the toxic properties of inactivated (i.e., rendered incapable of reproduction or germination) microbial pesticides. Inactivation should be done by means/methods that allows the maintenance of structural integrity of the microbial pesticides.

Dosing (Dose level)

A single dose level [at least 10^6 to 10^{10} colony forming units (CFU's) or viable spores or polyhedral occlusion bodies (POB's)], the active ingredient of the respective microbial pesticides per test animal is administered for the following categories:

i) Antagonistic bacteria/Antagonistic fungi/Entomopathogenic fungi based microbial pesticides:
A single dose level (at least 10^6 to 10^9 CFU's) of the microbial pesticides per test animal is administered.

ii) ***Entomotoxic bacteria based microbial pesticides:***
A single dose level (at least 10^6 to 10^9 viable spore count) of the microbial pesticides per test animal is administered.

iii) ***Baculoviruses based microbial pesticides:***
A single dose level of (at least 10^8 to 10^{10} POB's) of the microbial pesticides per test animal is administered.

Method of dose administration

i) Overnight fasted animals are administered a single dose of test substance by oral gavage using a stomach tube or suitable intubation cannula. After the administration of test substance, food is withheld for next 4 hours.

ii) If a single dose is not possible to administer, the dose may be given in smaller portions over a period not exceeding 24 hours. In such situations, it may become necessary to provide the animals with food and water,

iii) The maximum volume of liquid that can be administered per orally at one time depends on the size of the test animal. In rodents, the volume should not exceed 2ml/100gm of body weight. Variability in test volume should be minimized.

Observation period

After dosing the animals (test/control) should be observed for a period of at least 21 days. However, the duration of observation should be determined by the type of microbial pesticide administered and its rate of clearance from the test animals. Duration of the observation period would also depend on the time at which signs of toxicity and pathology appear and disappear, and the time of death of the animals.

Experimental observations

i) Observations for any clinical signs of toxicity/pathogenicity should be made on every animal, at least once during the first 30 minutes of dosing, followed by periodic observations for first 24 hours (special attention for the first 4 hours), thereafter at least once daily for the whole observation period. The onset and duration of toxicity/pathogenicity signs, if any, should be observed and recorded.

ii) Cage-side observations should include, but not limited to, change in condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous systems, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

iii) Weight of the individual test animals should be recorded, shortly prior to the administration of test substance, weekly (at least) after the dosing and at the time of death or interim or final sacrifice. All surviving animals are to be weighed before they are humanely sacrificed.

iv) Animals should be observed meticulously twice daily throughout the observation period for signs and symptoms of toxicity or mortality, if any. If any experimental animal is found in a moribund condition or showing signs of severe distress should be humanely sacrificed.

v) The rectal temperature of each animal should be recorded in the morning and evening, daily, throughout the observation period.

vi) Clinical pathology: Blood sample should be collected from orbital sinus of all the animals on day 0 and 21 of initiation of experiment (not to be evaluated on interim sacrifice & inactivated test group) and tested for RBC Count, WBC Count, haemoglobin, haematocrit, differential count, prothrombin time, clotting time, glucose, total protein, albumin, alanine aminotransferase, aspartate transaminase and blood urea nitrogen.

vii) Gross Pathology: All test animals should be subjected to gross necropsy at the time of death or interim or final sacrifice to record gross pathological findings.

viii) Organ Weights: Brain, heart, liver, lungs, spleen, adrenals, kidneys, gonads, trimmed of fascia and adipose adherents should be collected and their lean weights should be recorded.

Rate of Clearance of test substance (microbial pesticide)

Faeces from test animals should be collected soon after oral administration of microbial pesticide and then frequently during the study period. Stool is examined for the presence of the microbial pesticides and for its clearance. CFU/Viable spore count of the test microbial pesticide for Antagonistic bacteria/Antagonistic fungi/Entomopathogenic fungi & Entomotoxic bacteria based Microbial Pesticide respectively should be enumerated using selective culture medium technique. Whereas, in case of Baculoviruses based microbial pesticides, the presence of viable infective POB's should be detected using most sensitive respective larvae bioassay.

Infectivity evaluation

For infectivity and persistence determinations, the microbial pesticide should be enumerated using selective culture medium technique for its presence in tissues, organs, body fluids, lesions and the injection site. Homogenized samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces need to be cultured on selective medium to determine the infectivity/pathogenicity for Antagonistic bacteria/ Antagonistic fungi/Entomopathogenic fungi & Entomotoxic bacteria based microbial pesticide, whereas, in case of Baculoviruses based microbial pesticides, the presence of viable infective POB's should be detected using most sensitive respective larvae bioassay.

Interim sacrifice

For evaluating infectivity and clearance, the microbial pesticides should be enumerated from tissues, organs, and body fluids of three treated animals per gender, sacrificed at day 3, and at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to establish a pattern of clearance adequately. The microbial pesticide isolated from the tissues, organs, and body fluids of the *''shelf control''* group at final sacrifice should be enumerated.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity, rate of clearance and mortality as the study end points.

Cautionary Statement

If any microbial pesticide shows infectivity or unusual persistence or significant signs of acute oral toxicity/pathogenicity etc. in any of the rodent species, the applicant should contact/consult toxicology expert of CIB&RC for further testing requirements.

Reference: OPPTS 885.3050; Acute Oral Toxicity/Pathogenicity

Acute Pulmonary (Toxicity/Infectivity/Pathogenicity) Study

Objective: To assess and evaluate the toxic or pathogenic characteristics of a Microbial Pesticide.

Purpose: The purpose of the acute pulmonary toxicity study is to provide initial information on the toxicity, infectivity, and/or pathogenicity of microbial pesticides using a single dose exposure for prescribed observation period. It provides information on health hazards likely to arise from a single exposure by the pulmonary route in small laboratory animals.

Principle/Rationale: The test Microbial Pesticide is instilled/administered intranasally/intratracheally in a single dose to experimental animals. Subsequent observations of effects and deaths are made and rate of clearance of the Microbial Pesticides is estimated. Animals that die during the test are necropsied, and at the end of the test the surviving animals are sacrificed and necropsied ethically. Infectivity of the Microbial Pesticides is evaluated periodically during the test, and at the end of the test.

Test animals

Species: Rat is the recommended rodent species. Commonly used laboratory strains should be selected. If another species is used, justification/reasoning for the alternative selection should be provided. All test animals should be free of parasites or pathogens and experiments must be conducted as per Good Laboratory Practices (GLP) and CPCSEA guidelines.

Age: Between 8 and 12 weeks at the time of dosing.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Gender: Equal numbers of animals of each gender (sex) are required.

Housing and Feeding Conditions

Temperature: 22°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Groups and numbers of animals required

Test group

At least 10 animals (5 animals of each gender) should be used. Females should be nulliparous and non pregnant. A sufficient number of additional animals should be taken for interim sacrifice to determine infectivity.

Control group

i) A concurrent "untreated control" group of four animals per gender is required. Half of the animals in the control group (i.e. two animals per gender) should be housed sepaprately from the test group of animals dosed with microbial pesticides. The remainder of control group (shelf control) animals (comprising of two males & females) should be housed with the dosed/treated animals to evaluate infectivity of test compound.

ii) A separate "vehicle control" group is not required except in situations where the toxicity of the vehicle is not known.

iii) An additional control group including two males &two females are dosed to prove/ evaluate the toxic properties of inactivated (i.e., rendered incapable of reproduction or germination) microbial pesticides. Inactivation should be done by means/methods that allows the maintenance of structural integrity of the microbial pesticides.

Dosing (Dose level)

A single dose level of at least 10^8 Colony Forming Units (CFU) or 2 gm/ kg body weight of the Microbial Pesticides per test animal should be used.

Vehicle

The recommended vehicle for the pesticides is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the Microbial pesticides will be distributed, mixed, suspended, or diluted for application.

Administration method

Volume

The maximum volume of liquid that can be administered via intranasal or intratracheal routes at one time depends on the size of the test animal. In rat, the volume usually should not exceed 0.3 mL/100 g body weight. Variability in test volume should be minimized.

Dose quantification

Techniques used to quantify the units of microbial pesticides in any dose will depend on the group of microorganisms to which the Microbial pesticides belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. Quantification should be done concurrently with testing.

Exposure

The test substance should be administered via an appropriate delivery system in a single dose into the trachea of each test animal, or by intranasal instillation.

Observation

After dosing the animal (dosed/control) should be observed for a period of at least 21 days. However, the duration of observation should be determined by the type of microbial pesticide administered and its rate of clearance from the test animals. Duration of the observation period also would depend on the time at which signs of toxicity and pathology appear and disappear, and the time of death of the animals. The following parameters should be observed:

i) Observations should be made on individual animals at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), thereafter at least once daily during the whole observation period.

ii) Observations should include condition of skin and fur, eyes and mucus membrane, respiratory, circulatory, autonomic and central nervous systems, somato-motor activity and behavioural pattern. Specific observations should be made for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

iii) The rectal temperature of each animal will be recorded in the morning and evening, daily, throughout the observation period.

iv) Weight of the individual test animals should be recorded, shortly prior to the administration of test substance, weekly (at least) after the dosing and at the time of death or interim or final sacrifice. All surviving animals are to be weighed before they are humanely sacrificed.

v) Animals found in a moribund condition or showing signs of severe distress will be humanely sacrificed.

vi) All test animals should be subjected to gross necropsy at the time of death or interim or final sacrifice to record gross pathological findings. If any sign and /or symptom is suggestive, histopathology, biochemical and haematological parameters may be done.

Rate of Clearance of test substance (microbial pesticide)

Lungs from test animals sacrificed should be examined for the presence of the microbial pesticides to estimate clearance after administration. The Recovery values, detection and sensitivity limits of test substance (microbial pesticide) should be done using appropriately sensitive analytic method.

Infectivity evaluation

For infectivity and persistence determinations, the microbial pesticide should be enumerated by using sensitive techniques for its presence in tissues, organs, body fluids, lesions and from the injection site. Homogenized samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces need to be cultured on selective medium to determine the infectivity/Pathogenicity.

Interim sacrifice

For evaluating infectivity and clearance, the microbial pesticides should be enumerated from tissues, organs, and body fluids of three treated animals per gender, sacrificed at 3 days after, and at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to establish a pattern of clearance adequately. The microbial pesticide isolated from the tissues, organs, and body fluids of the ''shelf control'' group at final sacrifice should be enumerated.

Result assessment

The safety of the test substance (microbial pesticide) should be based on the observations for its infectivity, pathogenicity, toxicity, rate of clearance and mortality as the study end points.

Reference: OPPTS 885.3150; Acute Pulmonary toxicity/pathogenecity

Cell Culture Study

Objective: To assess and evaluate the toxic, infective, replicative and transformation characteristics of a Microbial Pesticide in the test cell culture.

Purpose: The purpose ofCell culture tests is to provide information on the ability of viral pest control agents to infect, replicate in, transform or cause toxicity in, mammalian cell lines.

Substance to be tested

The purest, most infectious (MIF) of the virus should be used. Preparations of insect viruses should be free of insect hemolymph, unless it has been determined that the hemolymph is not toxic to the cell cultures used. The inoculum should be titered by the most sensitive assay available, and in the most permissive host system (cell culture or, if not available, host organism). For testing in the model systems, a minimum of five plaque-forming units (PFU) per cell is required when a plaque assay for the virus is available, or 7× theLD50 units when a plaque assay for the virus is not available. If fewer units per cell or organism are used, justification/reasoning must be provided for using the lesser amount.

Cell cultures

The following cells are recommended—one human line (e.g., WI38), one primary cell type (e.g. foreskin), one primate continuous line (e.g. monkey CV-1 or BSC-1), primary Syrian hamster embryo (SHE) cells (to provide data for the cell transformation assay, described under paragraph (d)(5) of this guideline). One other cell line is to be selected to evaluate potential concerns intrinsic to the specific viral pest control agent, and its intended use. Justification/reasoning must be provided for the selection of this latter cell line.

Toxicity evaluation

Efficiency of plating tests should be performed with each cell line. For each cell line, approximately 200 cells are plated on each of 30 dishes. At 24 h postplating, 10 dishes per cell line are exposed to approximately 106 units of the virus. Appropriate vertebrate cell culture medium is added to 10 different dishes, and, if applicable, 10 dishes per cell line are exposed to invertebrate medium only. At 1 h postexposure, all cultures are fed with the appropriate vertebrate cell culture medium, and are incubated until control cultures have colonies consisting of at least 25 cells/colony. All cultures are fixed and stained, and colonies are enumerated.

Infectivity evaluation

i) Subconfluent cultures (containing approximately 2 × 105 cells on 25 cm2 dishes) of each cell line are exposed to >l × 106 units of the viral pest control agent. Appropriate controls include subconfluent cultures that receive no treatment, and those that are exposed to virus-free inoculation medium. At 7 days and at 14 days postinoculation, cells are to be subcultured.

ii) Cell cultures are observed daily for 21 days postinoculation for appearance of CPE.

iii) Cultures are quantitatively assayed for the virus on days 1, 2, 5, 7, 14, and 21 postinoculation.

A) Cells (entire culture, or >2 × 105 cells) are to be assayed in triplicate for viral antigen and nucleic acid.
B) Cell culture fluid from replicate cultures is to be assayed for infectious virus, using an appropriate susceptible host model system.

iv) Assays for fate of input virus and for presence of viral proteins and nucleic acid.
The enzyme linked immunosorbent assay (ELISA), dotimmunobinding assay, protein blot immunoassay,dot hybridization procedure, Southern hybridization procedure or similar assays are recommended for protein determination.

(v) To serve as controls, for each cell culture, cells inoculated with a preparation of the inactivated test virus should be analyzed as described for the active test virus, and for each series of tests, the inoculum should be tested in the permissive cell line or host organism as a positive control and for direct reference to the data obtained from the vertebrate cell lines.

Cell transformation assay

i) The ability of the viral pest control agent to transform primary Syrian hamster embryo (SHE) cells is to be determined, using an appropriate assay system. If other test systems are used, justification/rationale must be presented to show that the alternate systems are appropriate.

ii) Transformation of SHE cells with Simian adenovirus 7 (SAV 7) serves as the positive control. SHE cells treated with cell culture medium alone, and SHE cells treated with a killed preparation of the inactivated viral pest control agent serve as appropriate negative controls. The inactivation procedure must be demonstrated as effective in preventing transformation. An efficiency of plating test with SHE cells is considered an appropriate toxicity control.

iii) If the data show that the test virus modifies the cell phenotype, cells from cultures derived from morphologically transformed colonies are to be inoculated into hamsters, and tumorigenesis in the host animal is to be evaluated.

iv) This assay may not be required if, in the infectivity evaluations it is conclusively demonstrated that viral nucleic acid is not persistent in any of the test cell lines employed.

Evaluations

The following information should be provided for each test:

CPE in the cell monolayers

i) The appearance of CPE should be described in such a way that virus-induced cell destruction is differentiated from nonspecific effects.

ii) Cultures should be inspected with the aid of a microscope to provide evidence of CPE that should be recorded as:

(A) 1+ = suggestive of virus-induced morphologic changes.
(B) 2+ = definitive morphologic changes.
(C) 3+ = more than 50 percent cell degeneration.
(D) 4+ = complete cell destruction.

iii) The TCID50 value calculated by an appropriate statistical method. For computation of the infectivity results, only cultures showing a >2+ CPE are considered to be infected.

Toxicity evaluation

i) Details of all procedures used, including appropriate reagents and materials, and assay sensitivities and limitations.

ii) Efficiency of plating data of cultures receiving virus, and cultures receiving vertebrate media (control cultures) and invertebrate media.

iii) Assessment of mitotic process prevention or of interference with chromosomal replication, as indicated for example, by significant reductions in efficiency of plating.

Assay of culture fluid: Details of procedures used, including a discussion of all data that indicate viral replication.

Data from assays of input viruses

i) Details of procedures used for detection of viral antigens and nucleic acids and their persistence in culture, including appropriate reagents and materials, and assay sensitivities and limitations.

ii) Intracellular concentration of viral antigens and viral nucleic acids, reported as a function of cell number (e.g., viral genome number/ cell).

Cell transformation assay

i) Details of the protocols used for the cell transformation assay.

ii) Control value data, including deficiency of plating results.

General information to be provided for all tests

i) The source of each cell line used.

ii) Evidence for lack of adventitious agents in cell lines.

iii) Information on genetic stability of continuous cell lines, and on donors of primary cells.

Reference: OPPTS 885.3500; Cell culture

Acute Eye irritation study

Objective: To asses and evaluate the toxic, irritant and/or corrosive effects of microbial pesticide on eyes of mammals.

Purpose: The purpose of the acute eye irritation study is to provide initial information on the irritant and corrosive effects of microbial pesticides using a single dose exposure for prescribed observation period. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

Principle/Rationale: The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specified intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed. The period of observation should be at least 72 h, but need not exceed 21 days. Animals showing severe and enduring signs of distress and pain may need to be killed in a humane fashion.

Initial considerations

1) Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested owing to their predictable corrosive properties. Buffer capacity should also be taken into account.

2) Materials which have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation. It may be presumed that such substances will produce similarly severe effects in the eyes.

3) Results from well validated and accepted *in vitro* test systems may serve to identify corrosives or irritants such that the test material need not be tested *in vivo*.

Test animals

Species: The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

Age: Young adult animals should be used

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Housing and Feeding Conditions
Temperature: 22°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle

Diet and water:*Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization:The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Numbers of animals required

Treated group

A single animal should be considered if marked effects are anticipated. If the results of this test in one animal suggest the test substance to be a severe irritant (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further tests may not need to be performed. In cases other than a single animal test, at least three animals should be used. Occasionally, further testing in additional animals may be appropriate to clarify equivocal responses.

Control group

Separate animals are not recommended for an untreated control group. Untreated eye of each animal may serve as a control for the test.

Dose level

For testing liquids, a dose of 0.1 mL is recommended. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0.1 mL, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them (e.g. by tapping the measuring container). To test a substance contained in a pressurized aerosol container, the eye should be held open and the test substance administered in a single burst of about 1 sec from a distance of 10 cm directly in front of the eye. The dose may be estimated by weighing the container before and after use. Care should be taken not to damage the eye. Pump sprays should not be used but instead the liquid should be expelled and 0.1 mL collected and instilled into the eye as described for liquids. For volatile substances, the dose may be estimated by weighing the container before and after use.

Dosing (Dose level)

One dose level of at least 10^9 Colony Forming Units (CFU) or 2 gm/ kg body weight of the Microbial Pesticides per test animal should be used.

Examination of eyes prior to test

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 h before testing starts by the same procedure to be used during the test examination. Animals showing eye irritation, ocular defects, or preexisting corneal injury should not be used.
Application of the test substance

i) The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about 1 sec in order to limit loss of the material. The other eye, which remains untreated, serves as a control. If it is thought that the substance may cause extreme pain, local anesthetic may be used prior to instillation of the test substance. The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized.

ii) The eyes of the test animals should not be washed out for 24 h following instillation of the test substance. At 24 h, a washout may be used if considered appropriate. This is to show whether washing with water palliates or exacerbates irritation.

(iii) For some substances shown to be irritating by this test, additional testing using animals with eyes washed soon after instillation of the substance may be indicated. Half a minute after instillation, the eyes of the animals are washed with water for 30 sec, using a volume and velocity of flow which will not cause injury.

Observation period

The duration of the observation period is at least 72 h, and should not be fixed rigidly, but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. The observation period normally need not exceed 21 days after instillation.

Clinical examination and scoring

i) The eyes should be examined at 1, 24, 48, and 72 h. If there is no evidence of irritation at 72 h, the study may be ended. Extended observation (e.g. at 7 and 21 days) may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the cornea, iris and conjunctivae, any other lesions which are noted should be recorded and reported. The grades of ocular reaction using the following table should be recorded at each examination.

ii) Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 h, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

iii) The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used.

Data summary

Data should be summarized in tabular form, showing for each individual animal the irritation scores at observation time up until reversal (nonpositive grades) or 21 days when the test is concluded; a description of the degree and nature of irritation; the presence of serious lesions and any effects other than ocular which were observed.

Evaluation of the results

The ocular irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

Grades for Ocular Lesions	
Cornea	
Opacity: Degree of density (area most dense taken for reading). No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	*1
Easily discernible translucent area, details of iris slightly obscured	*2
Nacrous area, no details or iris visible, size of pupil barely discernible	*3
Opaque cornea, iris not discernible through the opacity	*4
Iris	
Normal	0
Markedly deepened rugae, congestion, swelling moderate circumcorneal hyperemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	*1
No reaction to light, hemorrhage, gross destruction (any or all of these)	*2
Conjunctivae	
Redness (refers to palpebral and bulbar conjunctivae, excluding cornea and iris)	
Blood vessels normal	0
Some blood vessels definitely hyperemic (injected)	1
Diffuse, crimson color, individual vessels not easily discernible	*2
Diffuse beefy red	*3
Chemosis (refers to lids and/or nictating membranes)	
No swelling	0
Any swelling above normal (includes nictating membranes)	1
Obvious swelling with partial eversion of lids	*2
Swelling with lids about half closed	*3
Swelling with lids more than half-closed	*4

*Starred figures indicate positive grades.

Result assessment

The eye irritation caused by the test substance (microbial pesticide) should be based on the observations of ocular lesions as the study end points.

Reference: OPPTS 870.2400; Acute Eye Irritation

PROPOSED TOXICOLOGICAL GUIDELINES/DATA REQUIREMENTS FOR REGISTRATION OF ANTAGONISTIC BACTERIA BASED BIO-PESTICIDES UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968

(w.e.f. 1st January, 2021)

I. STANDARD OF FORMULATIONS:

1 Colony Forming Unit (CFU) count on selective medium should be minimum of 1 x 10^8 per ml or gm for antagonistic bacteria.

2. **Contaminants:**
   - 2.1 Biological contaminants:
     - 2.1.1 Pathogenic contaminants such as gram negative bacteria (*Salmonella, Shigella, Vibrio)* and other microbials should not be present.
     - 2.1.2 Non-pathogenic microbial contaminants should not exceed 1 x 10^4 count per ml or per g of formulation.
   - 2.2 Chemical/botanical pesticide contaminants should not be present.

3. Stability of CFU counts at 30°C and 65% RH.

II. REGISTRATION REQUIREMENTS:

S. No.	STUDY PARTICULARS	9(3B)	9(3)
A.	**SINGLE EXPOSURE STUDIES ON PRIMARY CULTURE/MOTHER CULTURE (TECHNICAL)**		
i)	Single Dose Oral – (Rat & Mouse) (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	Single Dose Dermal – Rabbit (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	Single Dose Pulmonary* – Rat (Toxicity/Infectivity/Pathogenicity)	R	R
iv)	Single Dose Intraperitoneal – Rat (Toxicity/Infectivity/Pathogenicity)	R	R
v)	Human Safety Records (Effect/Lack of effects)	NR	R
B.	**SINGLE EXPOSURE STUDIES ON FORMULATION/FORMULATED PRODUCT**		
i)	Single Dose Oral – Rat (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	Single Dose Pulmonary* – Rat (Toxicity/Infectivity/Pathogenicity)	R	R

iii)	Primary Skin Irritation-Rabbit	R	R
iv)	Primary Eye Irritation- Rabbit	R	R
v)	Skin Sensitization-Guinea pig	R	R
vi)	Human Safety Records (Effect/Lack of effects)	NR	R
C.	**ENVIRONMENTAL SAFETY TESTING: ECO-TOXICITY STUDIES ON FORMULATION/FORMULATED PRODUCT**		
I	**Non-target Vertebrates**		
i)	Toxicity to Chicken (Toxicity/Infectivity/Pathogenicity)	NR	R
ii)	Toxicity to Pigeon (Toxicity/Infectivity/Pathogenicity)	NR	R
iii)	Toxicity to Fresh water Fish [a] (Toxicity/Infectivity/Pathogenicity)	NR	R
II	**Non-target Invertebrates**		
i)	Toxicity to Honey bee (Terrestrial Invertebrates)	NR	R
ii)	Toxicity to Silk worm (Terrestrial Invertebrates)	NR	R
iii)	Toxicity to Earth worm [b] (Soil invertebrates)	NR	R

Notes:

Abbreviations: R-Required; NR-Not Required;

* Intranasal/Intratracheal, if formulation does not permit intratracheally, then to go for intranasal.

[a] = Information on infection and pathogenicity: suggested test species: *Tilapia mossambica* or other appropriate spp.

[b] = Information on mortality effects. It is recommended that test species include an earthworm (*Lumbricus terrestris*) or other appropriate macro invertebrates of ecological significance.

1. One of the category mentioned in the existing guidelines, "*Formulated Product to be directly manufactured*" was introduced in the year 2002 with a motive to promote bio-pesticides without asking for the toxicological data on primary culture. However, as the

toxicological data on primary culture is now mandatory, the said category appears to be redundant in current scenario, hence deleted.

2. (i) Since Single Dose Oral Toxicity/Infectivity/Pathogenicity study is recommended on primary/mother culture with higher concentration of active ingredient (i.e. CFU count) on two species (rat and mouse), therefore, in case of formulation only in one species Single Dose Oral Toxicity/Infectivity/Pathogenicity (rat) may suffice as the colony count is lower than that has already been tested in the mother culture.

   (ii) To ensure the dermal safety, further Skin Sensitization test is proposed to include as bacterias are proteinaceous material, hence, prone to cause hypersensitivity.

3. If the isolate is to be imported, then in that case, additional acute oral mouse study shall be applicable.

4. If same microbial strain is used for making formulation by different entrepreneurs, then the information submitted once by the original (first) registrant on the said strain will be sufficient. All entrepreneurs need not generate data, however, the subsequent firm/applicant shall submit the permission along with authentication/authorization letter from the original registrant of the strain and for use of data dossier.

5. If the applicant is submitting the Direct-9(3) application in that case undertaking is required for Health monitoring studies (Human safety records) of workers engaged in manufacturing of primary culture & formulation of Bio-pesticide product to be submitted within two years from the date of issue of registration certificate. In case of non-compliance of the conditions, the certificate of registration shall be deemed invalid. For category 9(3B) to 9(3) registration, copy of all the toxicological data submitted at the time of 9(3B) is also required in 9(3) registration dossier.

6. All the toxicity studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non-Guideline compliant studies will be considered on their scientific merit as additional information/study.

PROPOSED TOXICOLOGICAL GUIDELINES/DATA REQUIREMENTS FOR REGISTRATION OF ANTAGONISTIC FUNGI BASED BIO-PESTICIDES UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968

(w.e.f. 1st January, 2021)

I. STANDARD OF FORMULATIONS:

1 Colony Forming Unit (CFU) count on selective medium should be minimum of 2 x 10^6 per ml or gm for antagonistic fungi.

2. **Contaminants:**

2.1 Biological contaminants:

2.1.1 Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and other microbials should not be present.

2.1.2 Non-pathogenic microbial contaminants should not exceed 1 x 10^4 count per ml or per g of formulation.

2.2 Chemical/botanical pesticide contaminants should not be present.

3. Stability of CFU counts at 30°C and 65% RH.

II. REGISTRATION REQUIREMENTS:

S.No.	STUDY PARTICULARS	9(3B)	9(3)
A.	**SINGLE EXPOSURE STUDIES ON PRIMARY CULTURE/MOTHER CULTURE (TECHNICAL)**		
i)	**Single Dose Oral – (Rat & Mouse)** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Dermal – Rabbit** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Single Dose Pulmonary* – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iv)	**Single Dose Intraperitoneal – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
v)	**Human Safety Records** (Effect/Lack of effects)	NR	R
B.	**SINGLE EXPOSURE STUDIES ON FORMULATION/FORMULATED PRODUCT**		
i)	**Single Dose Oral – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R

ii)	**Single Dose Pulmonary* – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Primary Skin Irritation-Rabbit**	R	R
iv)	**Primary Eye Irritation- Rabbit**	R	R
v)	**Skin Sensitization-Guinea pig**	R	R
vi)	**Human Safety Records** (Effect/Lack of effects)	NR	R
C.	**ENVIRONMENTAL SAFETY TESTING: ECO-TOXICITY STUDIES ON FORMULATION/FORMULATED PRODUCT**		
I	**Non-target Vertebrates**		
i)	**Toxicity to Chicken** (Toxicity/Infectivity/Pathogenicity)	NR	R
ii)	**Toxicity to Pigeon** (Toxicity/Infectivity/Pathogenicity)	NR	R
iii)	**Toxicity to Fresh water Fish[a]** (Toxicity/Infectivity/Pathogenicity)	NR	R
II	**Non-target Invertebrates**		
i)	**Toxicity to Earthworm[b]** (Soil invertebrates)	NR	R

Notes:

Abbreviations: R-Required; NR-Not Required;

* Intranasal/Intratracheal, if formulation does not permit intratracheally, then to go for intranasal.

[a] = Information on infection and pathogenicity: suggested test species: *Tilapia mossambica* or other appropriate spp.

[b] = Information on mortality effects. It is recommended that test species include an earthworm (*Lumbricus terrestris*) or other appropriate macro invertebrates of ecological significance.

1. One of the category mentioned in the existing guidelines, "*Formulated Product to be directly manufactured*" was introduced in the year 2002 with a motive to promote bio-pesticides without asking for the toxicological data on primary culture. However, as the toxicological data on primary culture is now mandatory, the said category appears to be redundant in current scenario, hence deleted.

2. (i) Since Single Dose Oral Toxicity/Infectivity/Pathogenicity study is recommended on primary/mother culture with higher concentration of active ingredient (i.e. CFU count) on two species (rat and mouse), therefore, in case of formulation only in one species Single Dose Oral Toxicity/Infectivity/Pathogenicity (rat) may suffice as the colony count is lower than that has already been tested in the mother culture.

   (ii) To ensure the dermal safety, further Skin Sensitization test is proposed to include as fungi are proteinaceous material, prone to cause hypersensitivity.

3. If the isolate is to be imported, then in that case, additional acute oral mouse study shall be applicable.

4. If same microbial strain is used for making formulation by different entrepreneurs then the information submitted once by the original (first) registrant on the said strain will be sufficient. All entrepreneurs need not generate data, however, the subsequent firm/applicant shall submit the permission along with authentication/authorization letter from the original registrant of the strain and for use of data dossier.

5. If the applicant is submitting the Direct-9(3) application in that case undertaking is required for Health monitoring studies (Human safety records) of workers engaged in manufacturing of primary culture & formulation of Bio-pesticide product to be submitted within two years from the date of issue of registration certificate. In case of non-compliance of the conditions, the certificate of registration shall be deemed invalid. For category 9(3B) to 9(3) registration, copy of all the toxicological data submitted at the time of 9(3B) is also required in 9(3) registration dossier.

6. All the toxicity studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non-Guideline compliant studies will be considered on their scientific merit as additional information/study.

PROPOSED TOXICOLOGICAL GUIDELINES/DATA REQUIREMENTS FOR REGISTRATION OF BACULOVIRUSES - NUCLEAR POLYHEDROSYS VIRUS (NPV) & GRANULOSIS VIRUS (GV) UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968

(w.e.f. 1st January, 2021)

I. STANDARD OF FORMULATIONS:

1 Polyhedral Occlusion Bodies (POB) count:
NPVs $1x10^9$ POB per ml or gm (minimum)
GVs: $5x10^9$ capsules per ml or gm (minimum)

2. **Contaminants:**
   - 2.1 Biological contaminants:
   - 2.1.1 Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and other microbials should not be present.
   - 2.1.2 Non-pathogenic microbial contaminants should not exceed 1×10^4 count per ml or per g of formulation.
   - 2.2 Chemical/botanical pesticide contaminants should not be present.

I. REGISTRATION REQUIREMENTS:

S.No.	STUDY PARTICULARS	9(3B)	9(3)
A.	**SINGLE EXPOSURE STUDIES ON PRIMARY CULTURE/MOTHER CULTURE (TECHNICAL)**		
i)	**Single Dose Oral – (Rat & Mouse)** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Pulmonary* – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Single Dose Intravenous – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iv)	**Cell Culture**	R	R
v)	**Human Safety Records** (Effect/Lack of effects)	NR	R
B.	**SINGLE EXPOSURE STUDIES ON FORMULATION/FORMULATED PRODUCT**		
i)	**Single Dose Oral – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Pulmonary* – Rat**	R	R

	(Toxicity/Infectivity/Pathogenicity)		
iii)	**Primary Skin Irritation-Rabbit**	R	R
iv)	**Primary Eye Irritation- Rabbit**	R	R
v)	**Skin Sensitization-Guinea pig**	R	R
vi)	**Human Safety Records** (Effect/Lack of effects)	NR	R
C.	**ENVIRONMENTAL SAFETY TESTING: ECO-TOXICITY STUDIES ON FORMULATION/FORMULATED PRODUCT**		
I	**Non-target Vertebrates**		
i)	**Toxicity to Chicken** (Toxicity/Infectivity/Pathogenicity)	NR	R
ii)	**Toxicity to Pigeon** (Toxicity/Infectivity/Pathogenicity)	NR	R
iii)	**Toxicity to Fresh water Fish**[a] (Toxicity/Infectivity/Pathogenicity)	NR	R
II	**Non-target Invertebrates**		
iv)	**Toxicity to Honey bee** (Terrestrial Invertebrates)	NR	R
v)	**Toxicity to Silk worm** (Terrestrial Invertebrates)	NR	R
vi)	**Toxicity to Earthworm**[b] (Soil invertebrates)	NR	R

Notes:

Abbreviations: R-Required; NR-Not Required;

* Intranasal/Intratracheal, if formulation does not permit intratracheally, then to go for intranasal.

[a] = Information on infection and pathogenicity: suggested test species: *Tilapia mossambica* or other appropriate spp.

[b] = Information on mortality effects. It is recommended that test species include an earthworm (*Lumbricus terrestris*) or other appropriate macro invertebrates of ecological significance.

1. One of the category mentioned in the existing guidelines, "Formulated Product to be directly manufactured" was introduced in the year 2002 with a motive to promote bio-

pesticides without asking for the toxicological data on primary culture. However, as the toxicological data on primary culture is now mandatory, the said category appears to be redundant in current scenario, hence deleted.

2. (i) Since Single Dose Oral Toxicity/Infectivity/Pathogenicity study is recommended on primary/mother culture with higher concentration of active ingredient (i.e. POBs count) on two species (rat and mouse), therefore, in case of formulation only in one species Single Dose Oral Toxicity/Infectivity/Pathogenicity (rat) may suffice as the colony count is lower than that has already been tested in the mother culture.

   (ii) To ensure the dermal safety, further Skin Sensitization test is proposed to include as virus are proteinaceous material, prone to cause hypersensitivity.

3. If the isolate is to be imported, then in that case, additional acute oral mouse study shall be applicable.

4. If same microbial strain is used for making formulation by different entrepreneurs then the information submitted once by the original (first) registrant on the said strain will be sufficient. All entrepreneurs need not generate data, however, the subsequent firm/applicant shall submit the permission along with authentication/authorization letter from the original registrant of the strain and for use of data dossier.

5. If the applicant is submitting the Direct-9(3) application in that case undertaking is required for Health monitoring studies (Human safety records) of workers engaged in manufacturing of primary culture & formulation of Bio-pesticide product to be submitted within two years from the date of issue of registration certificate. In case of non-compliance of the conditions, the certificate of registration shall be deemed invalid. For category 9(3B) to 9(3) registration, copy of all the toxicological data submitted at the time of 9(3B) is also required in 9(3) registration dossier.

6. All the toxicity studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non-Guideline compliant studies will be considered on their scientific merit as additional information/study.

PROPOSED TOXICOLOGICAL GUIDELINES/DATA REQUIREMENTS FOR REGISTRATION OF ENTOMOPATHOGENIC FUNGI BASED BIO-PESTICIDES UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968

(w.e.f. 1st January, 2021)

I. STANDARD OF FORMULATIONS:

1 Colony Forming Unit (CFU) count on selective medium should be minimum of 1 x 10^8 per ml or gm for entomopathogenic fungi.

2. **Contaminants:**
   - 2.1 Biological contaminants:
   - 2.1.1 Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and other microbials should not be present.
   - 2.1.2 Other non-pathogenic microbial contaminants should not exceed 1 x 10^4 count per ml or per g of formulation.
   - 2.2 Chemical/botanical pesticide contaminants should not be present.

3. Stability of CFU counts at 30°C and 65% RH.

II. REGISTRATION REQUIREMENTS:

S.No.	STUDY PARTICULARS	9(3B)	9(3)
A.	**SINGLE EXPOSURE STUDIES ON PRIMARY CULTURE/MOTHER CULTURE (TECHNICAL)**		
i)	**Single Dose Oral – (Rat & Mouse)** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Dermal – Rabbit** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Single Dose Pulmonary* – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iv)	**Single Dose Intraperitoneal – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
v)	Human Safety Records (Effect/Lack of effects)	NR	R
B.	**SINGLE EXPOSURE STUDIES ON FORMULATION/FORMULATED PRODUCT**		
i)	**Single Dose Oral – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Pulmonary* – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R

iii)	**Primary Skin Irritation-Rabbit**	R	R
iv)	**Primary Eye Irritation- Rabbit**	R	R
v)	**Skin Sensitization-Guinea pig**	R	R
vi)	**Human Safety Records** (Effect/Lack of effects)	NR	R
C.	**ENVIRONMENTAL SAFETY TESTING: ECO-TOXICITY STUDIES ON FORMULATION/FORMULATED PRODUCT**		
I	**Non-target Vertebrates**		
i)	**Toxicity to Chicken** (Toxicity/Infectivity/Pathogenicity)	NR	R
ii)	**Toxicity to Pigeon** (Toxicity/Infectivity/Pathogenicity)	NR	R
iii)	**Toxicity to Fresh water Fish**[a] (Toxicity/Infectivity/Pathogenicity)	NR	R
II	**Non-target Invertebrates**		
iv)	**Toxicity to Honey bee** (Terrestrial Invertebrates)	NR	R
v)	**Toxicity to Silk worm** (Terrestrial Invertebrates)	NR	R
vi)	**Toxicity to Earthworm**[b] (Soil invertebrates)	NR	R

Notes:

Abbreviations: R-Required; NR-Not Required;

* Intranasal/Intratracheal, if formulation does not permit intratracheally, then to go for intranasal.

[a] = Information on infection and pathogenicity: suggested test species: *Tilapia mossambica* or other appropriate spp.

[b] = Information on mortality effects. It is recommended that test species include an earthworm (*Lumbricus terrestris*) or other appropriate macro invertebrates of ecological significance.

1. One of the category mentioned in the existing guidelines, "*Formulated Product to be directly manufactured*" was introduced in the year 2002 with a motive to promote bio-

pesticides without asking for the toxicological data on primary culture. However, as the toxicological data on primary culture is now mandatory, the said category appears to be redundant in current scenario, hence deleted.

2. (i) Since Single Dose Oral Toxicity/Infectivity/Pathogenicity study is recommended on primary/mother culture with higher concentration of active ingredient (i.e. CFU count) on two species (rat and mouse), therefore, in case of formulation only in one species Single Dose Oral Toxicity/Infectivity/Pathogenicity (rat) may suffice as the colony count is lower than that has already been tested in the mother culture.

   (ii) To ensure the dermal safety, further Skin Sensitization test is proposed to be included as fungi are proteinaceous material, prone to cause hypersensitivity.

3. If the isolate is to be imported, then in that case, additional acute oral mouse study shall be applicable.

4. If same microbial strain is used for making formulation by different entrepreneurs then the information submitted once by the original (first) registrant on the said strain will be sufficient. All entrepreneurs need not generate data, however, the subsequent firm/applicant shall submit the permission along with authentication/authorization letter from the original registrant of the strain and for use of data dossier.

5. If the applicant is submitting the Direct-9(3) application in that case undertaking is required for Health monitoring studies (Human safety records) of workers engaged in manufacturing of primary culture & formulation of Bio-pesticide product to be submitted within two years from the date of issue of registration certificate. In case of non-compliance of the conditions, the certificate of registration shall be deemed invalid. For category 9(3B) to 9(3) registration, copy of all the toxicological data submitted at the time of 9(3B) is also required in 9(3) registration dossier.

6. All the toxicity studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non-Guideline compliant studies will be considered on their scientific merit as additional information/study.

PROPOSED TOXICOLOGICAL GUIDELINES/DATA REQUIREMENTS FOR REGISTRATION OF ENTOMOTOXIC/ENTOMOPATHOGENIC BACTERIA [TECHNICAL AND FORMULATION] UNDER SECTION 9(3B) and 9(3) OF THE INSECTICIDE ACT, 1968

(w.e.f. 1st January, 2021)

I. STANDARD OF FORMULATIONS:

1 Delta endotoxin content (Minimum 2.0%)
Beta Exotoin content – absent

2. **Contaminants:**
   - 2.1 Biological contaminants:
   - 2.1.1 Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and other microbials should not be present.
   - 2.1.2 Non-pathogenic microbial contaminants should not exceed 1 x 10^4 count per ml or per g of formulation.
   - 2.2 Chemical/botanical pesticide contaminants should not be present.

I. REGISTRATION REQUIREMENTS:

S.No.	STUDY PARTICULARS	9(3B)	9(3)
A.	**SINGLE EXPOSURE STUDIES ON TECHNICAL**		
i)	**Single Dose Oral – (Rat & Mouse)** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Dermal – Rabbit** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Single Dose Inhalation – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Primary Skin Irritation-Rabbit**	R	R
iv)	**Primary Eye Irritation- Rabbit**	R	R
v)	**Allergy/Skin Sensitization/Immunosuppression - Guinea pig**	R	R
iv)	**Human Safety Records** (Effect/Lack of effects)	NR	R
B.	**SINGLE EXPOSURE STUDIES ON FORMULATION/FORMULATED PRODUCT**		

i)	**Single Dose Oral – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
ii)	**Single Dose Dermal – Rabbit** (Toxicity/Infectivity/Pathogenicity)	R	R
iii)	**Single Dose Inhalation – Rat** (Toxicity/Infectivity/Pathogenicity)	R	R
iv)	**Primary Skin Irritation-Rabbit**	R	R
v)	**Primary Eye Irritation- Rabbit**	R	R
vi)	**Allergy/Skin Sensitization/Immunosuppression - Guinea pig**	R	R
vii)	**Human Safety Records** (Effect/Lack of effects)	NR	R
C.	**ENVIRONMENTAL SAFETY TESTING: ECO-TOXICITY STUDIES ON FOMULATION/FORMULATED PRODUCT**		
I	**Non-target Vertebrates**		
i)	**Toxicity to Chicken** (Toxicity/Infectivity/Pathogenicity)	NR	R
ii)	**Toxicity to Pigeon** (Toxicity/Infectivity/Pathogenicity)	NR	R
iii)	**Toxicity to Fresh water Fish**[a] (Toxicity/Infectivity/Pathogenicity)	NR	R
II	**Non-target Invertebrates**		
i)	**Toxicity to Honey bee**	NR	R
ii)	**Toxicity to Silk worm**	NR	R

Notes:

Abbreviations: R-Required; NR-Not Required;

[a] = Information on infection and pathogenicity: suggested test species: *Tilapia mossambica* or other appropriate spp.

1. (i) Since Single Dose Oral Toxicity/Infectivity/Pathogenicity study is recommended on primary/mother culture with higher concentration of active ingredient (i.e. Viable Spore count) on two species (rat and mouse), therefore, in case of formulation only in one species Single Dose Oral Toxicity/Infectivity/Pathogenicity (rat) may suffice as the colony count is lower than that has already been tested in the mother culture.

   (ii) To ensure the dermal safety, further Skin Sensitization test is proposed to include as bacterias are proteinaceous material, prone to cause hypersensitivity.

2. If the isolate is to be imported, then in that case, additional acute oral mouse study shall be applicable.

3. If same microbial strain is used for making formulation by different entrepreneurs then the information submitted once by the original (first) registrant on the said strain will be sufficient. All entrepreneurs need not generate data, however, the subsequent firm/applicant shall submit the permission along with authentication/authorization letter from the original registrant of the strain and for use of data dossier.

4. If the applicant is submitting the Direct-9(3) application in that case undertaking is required for Health monitoring studies (Human safety records) of workers engaged in manufacturing of primary culture & formulation of Bio-pesticide product to be submitted within two years from the date of issue of registration certificate. In case of non-compliance of the conditions, the certificate of registration shall be deemed invalid. For category 9(3B) to 9(3) registration, copy of all the toxicological data submitted at the time of 9(3B) is also required in 9(3) registration dossier.

5. All the toxicity studies must be conducted in Good Laboratory Practice (GLP) certified laboratory. The study reports should be accompanied with the GLP certification from the Study Director and Quality Assurance (QA) certificate from QA unit of the laboratory. Non-GLP or non-Guideline compliant studies will be considered on their scientific merit as additional information/study.

Acute dermal irritation study

Objective: To asses and evaluate the toxic, irritant and/or corrosive effects of microbial pesticide on skin of mammals.

Purpose: The purpose of acute dermal irritation study is to provide initial information on the irritant and/or corrosive effects of microbial pesticide on skin of mammals. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.

Principle/Rationale: The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control (except when severe irritation/corrosion is suspected and the stepwise procedure is used. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days. When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

Test animals

Species: The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

Age: Young adult animals should be used.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Housing and Feeding Conditions

Temperature: 22°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Numbers of animals required

Treated group

At least three healthy adult animals (either sex) should be used unless justification/reasoning for using fewer animals is provided. It is recommended that a stepwise procedure be used to expose

one animal, followed by additional animals to clarify equivocal responses.

Control group

Separate animals are not recommended for an untreated control group. Adjacent areas of untreated skin of each animal may serve as a control for the test.

Dosage

One dose level of at least 10^9 Colony Forming Units (CFU) or 2 gm/ kg body weight of the Microbial Pesticides per test animal should be used.

Administration method

Preparation of animal skin

i) Approximately 24 h before the test, fur should be removed from the dorsal and ventral area of the trunk of each test animal by clipping or shaving.

ii) Not less than 10 percent of the body surface area should be cleared for application of the test substance. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

Application of the test substance

i) The recommended exposure duration is normally 4 hours unless corrosion is observed. At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

ii) When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. If a vehicle is used, it should not alter the absorption, distribution, metabolism, retention or the chemical properties of the test substance nor should it enhance, reduce, or alter its toxic characteristics. Although water or saline is the preferred agent to be used for moistening dry test materials, other agents may be used providing the use is justified. Acceptable alternatives include: gum arabic, ethanol and water, carboxymethyl cellulose, polyethylene glycol, glycerol, vegetable oil, and mineral oil.

iii) The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with nonirritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semiocclusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

Observation

i)A careful clinical examination should be made at least once each day for 14 days.

ii) Cage side observations should include, but not be limited to, changes in skin (including signs of irritation) and fur, eyes and mucous membranes, Respiratory system, Circulatory system, Autonomic and central nervous system, Somatomotor activity, Behavior pattern, Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep, and coma.

iii) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30–60 min, and at 24, 48, and 72 hours after patch removal.

iv)Dermal irritation should be scored and recorded according to the grades in the following Table. 1. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.

v) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at final sacrifice. Changes in weight should be calculated and recorded when survival exceeds 1 day.

vi) At the end of the 24–hours exposure period, and daily thereafter, any signs of skin irritation should be recorded and scored.

Evaluation of Skin Reaction

	Value
Erythema and Eschar Formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	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 (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyong area of exposure	4
Maximum possible	4

Result assessment

The dermal safety of the test substance (microbial pesticide) should be based on the observations for its skin irritation as the study end points.

Reference: OPPTS 870.2500; Acute Dermal Irritation

Skin Sensitisation

Objective: To asses and evaluate the potential of microbial pesticide to elicit skin sensitization reactions in mammals.

Purpose: The purpose of skin sensitization study is to identify microbial pesticides with skin sensitization potential and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from skin sensitization study serves to identify possible hazards to a population exposed repeatedly to a test substance.

Any of the following test methods is considered to be acceptable for determination of skin sensitization:

i) Local Lymph Node Assay (LLNA) test, or
ii) Guinea-Pig Maximization Test (GPMT), or
iii) Buehler test.

Local Lymph Node Assay (LLNA method)

Principle of the method: The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application. Generally, under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of in vivo radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in concurrent solvent/vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

Test animals

Species: CBA/Ca or CBA/J strain of mice.

Age: Young adult animals aged 8-12 weeks should be used.

Gender: Animals of female gender (sex) can be used.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Housing and Feeding Conditions

Temperature: 21°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle

Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Numbers of animals required

Treated group

A minimum of five animals are used per dose group. At least three consecutive doses of the microbial pesticide are to be used. Doses are normally selected from within the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.1%.

Control group

i) Concurrent negative (solvent/vehicle) and positive controls are to be included in each test. In some circumstances, it may be useful to include a naive control.

ii) Positive controls are used to ensure the appropriate performance of the assay. The positive control must produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) of three or greater (SI $\geq$ 3) over the solvent or vehicle control group. The positive control dose is to be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used. However, benzocaine should not be used as a positive control in the LLNA.

iii) The positive control substance is tested in the vehicle that is known to elicit a consistent response (i.e., acetone/olive oil). If a nonstandard vehicle (chemically relevant formulation) is used with a positive control, the non-standard vehicle (chemically relevant formulation) must be tested for a local lymph node response prior to the initiation of the study and the results reported.

Administration method

i) Day 1. Record the body weight of each mouse prior to dermal applications. Apply 25 µL/ear of the appropriate dilution of the test substance, or the positive control, or the solvent/vehicle control alone to the dorsum of both ears. A positive displacement pipettor may facilitate application of the test material.

ii) Days 2 and 3. Repeat the application procedure as carried out on day 1.

iii) Days 4 and 5. No treatment.

iv) Day 6. Record the body weight of each mouse. Inject 250 µL of sterile phosphate buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine or 250 µL PBS containing 2 µCi ^{125}IU and 10^{-5} M fluorodeoxyuridine into each experimental mouse via the tail vein. Five hours later,

the draining (auricular) lymph node of each ear is excised and pooled in PBS for each animal. A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200 mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. The LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4 °C for approximately 18h.

v) For the ^{3}H-methyl thymidine method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of ^{3}H-methyl thymidine is measured by B-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the ^{125}IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

Observations

At a minimum, observe mice once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

Measurements and calculation of results

i) The proliferative response of lymph node cells from the pooled lymph nodes of each individual animal is expressed as the number of radioactive disintegrations per minute (dpm) per animal, subtracting out any background dpm. Then the group mean dpm, along with an appropriate measure of inter-animal variability (i.e., mean ± standard deviation), is calculated for each test group (i.e., positive, solvent/vehicle, and any other control groups) and the solvent/vehicle group. Final results are expressed as the SI which is calculated as a ratio (i.e., SI = mean dpm of test group divided by mean dpm of solvent/vehicle control group).

ii) In addition to an assessment of the magnitude of the ratio estimate, SI, conduct statistical analyses which include both an overall assessment (e.g. ANOVA) of the dose-response relationships and pair wise comparisons of the SIs of the test groups, positive control group and any other control group versus that of the solvent/vehicle control group. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

Data interpretation

i) A substance is regarded as a skin sensitizer in the LLNA if at least one concentration of the test material results in a 3-fold or greater increase in ^{3}H-methyl thymidine or ^{125}IU incorporation in the lymph node cells of test group lymph nodes relative to that recorded for solvent/ vehicle control lymph nodes, as indicated by the SI. However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment must be performed by statistical analysis of individual animal data in

order to provide a more complete evaluation of the test substance. Factors to be considered in evaluating the biological significance of a response or outcome of the test include the results of the SI determinations, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.
ii) Strong irritants may yield false positive results in the LLNA due to the initiation of a significant lymphocyte proliferation. However, the dose-response information from the assay may help to uncover a strong irritant response since, for instance, it has been shown that the proliferation induced by irritation usually results in a shallow dose-response relationship. Concurrent evaluation of ear swelling may also provide helpful information on differentiating weak sensitizers from strong irritants.

Guinea pig maximization test (GPMT) and Buehler test

Principle/Rationale: Following initial exposure to a test substance, the animals are subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of the initial induction exposure. The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure is compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure.

Test animals:

Species: The young adult guinea pig is preferred.

Age: Young adult animals should be used.

Gender: Animals of each gender (sex) can be used. If females are used, they must be nulliparous and not pregnant.

Weight: Weight variation should be within + 20 % of the mean ideal weight of the animal.

Housing and Feeding Conditions:
Temperature: 20°± 3°C
Relative Humidity: 50-60% (However, humidity should not be below 30% or exceed 70% at any given point of time).
Lighting: 12 hours light and dark cycle
Diet and water: *Ad libitum* standard diet, (specific to the species) and filtered water (free from contamination).It is essential that guinea pigs receive an adequate amount of ascorbic acid.
Acclimatization: The animals should be acclimatized to the laboratory conditions for at least 5 days by keeping in individual cages prior to dosing.

Preparation of animal skin

Hair is removed from the site of application by clipping, shaving, or possibly by depilation, depending on the test selected. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

Numbers of animals required

Either sex may be used. If females are used, they must be nulliparous and not pregnant. The Buehler test recommends using a minimum of 20 animals in the treatment and at least 10 as controls. At least 10 animals in the treatment group and 5 in the control group must be used with the GPMT, with the stipulation that if it is not possible to conclude that the test substance is a sensitizer after using fewer than 20 test and 10 control guinea pigs, the testing of additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

Control group

i) Every 6 months, assess the sensitivity and reliability of the experimental technique in naive animals by the use of positive control substances known to have mild-to-moderate skin-sensitizing properties. In a properly conducted test, a response of at least 30 percent in an adjuvant test and at least 15 percent in a nonadjuvant test is expected for mild-to-moderate sensitizers. Preferred substances are hexylcinnamic aldehyde, mercaptobenzothiazole, benzocaine, dinitro-chloro-benzene or DER 331 epoxy resin. There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

ii) To ensure that the response to the challenge reaction in treated animals is truly of allergic origin and not due to skin irritancy, a sham treated vehicle-only control is included in the test strategy. This sham treated control group is treated in exactly the same manner as the test animals, except that during the induction phase the test article is omitted. The selected vehicle must not interfere or alter the test results.

Dosage

In the Buehler test, select the concentration of the induction dose such that it is high enough to cause mild irritation, and the challenge dose such that it is the highest non-irritating concentration. In the GPMT, the concentration of the induction dose must be well tolerated systemically, and must be high enough to cause mild-to-moderate skin irritation; the GPMT challenge dose must use the highest non-irritating concentration

Administration method

The Buehler test uses topical administration via a closed patch on days 0, 6–8, and 13–15 for induction, with topical challenge of the untreated flank for 6 hours on day 27–28. Readings are made approximately 24 hours alter removing the challenge patch, and again 24 hours after that. If the results are equivocal, the animals may be rechallenged one week later, using either the

original control group or a new control group for comparison. The GPMT uses intradermal injection with and without Freund's complete adjuvant (FCA) for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20– 22. Readings are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours. As with the Buehler test, if the results are equivocal, the animals may be rechallenged 1 week later. If only 10 animals were used initially and gave equivocal results, the use of an additional 10 experimental and 5 control animals is strongly recommended. (Intradermal injection with and without Freund's complete adjuvant (FCA) for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20– 22. Readings are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours.

Removal of the test substance

If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Observations

i) Approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved or depilated if necessary;

ii) Approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown below;

iii) Approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded. Additional notations are to be made as necessary to fully describe unusual responses.

iii) Blind reading of test and control animals is encouraged.

iv) Initial and terminal body weights must be taken and recorded.

Clinical observations

All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures e.g. Histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

Evaluation of the results

i) Individual body weights of the animals at the start of the test and at the conclusion of the test.

ii) A brief description of the grading system:

0 = no visible change
1 = discrete or patchy erythema
2 = moderate and confluent erythema
3 = intense erythema and swelling

iii) Each reading made on each individual animal.

iv) Histopathological findings, if any.

Result assessment

The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

Reference: OPPTS 870.2600; Skin sensitization