

WHO SPECIFICATIONS AND EVALUATIONS

FOR PUBLIC HEALTH PESTICIDES

Bacillus thuringiensis subspecies israelensis
strain AM65-52



**World Health
Organization**

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Disclaimer¹

WHO specifications are developed with the basic objective of promoting, as far as practicable, the manufacture, distribution and use of pesticides that meet basic quality requirements.

Compliance with the specifications does not constitute an endorsement or warranty of the fitness of a particular pesticide for a particular purpose, including its suitability for the control of any given pest, or its suitability for use in a particular area. Owing to the complexity of the problems involved, the suitability of pesticides for a particular purpose and the content of the labelling instructions must be decided at the national or provincial level.

Furthermore, pesticides which are manufactured to comply with these specifications are not exempted from any safety regulation or other legal or administrative provision applicable to their manufacture, sale, transportation, storage, handling, preparation and/or use.

WHO disclaims any and all liability for any injury, death, loss, damage or other prejudice of any kind that may be arise as a result of, or in connection with, the manufacture, sale, transportation, storage, handling, preparation and/or use of pesticides which are found, or are claimed, to have been manufactured to comply with these specifications.

Additionally, WHO wishes to alert users to the fact that improper storage, handling, preparation and/or use of pesticides can result in either a lowering or complete loss of safety and/or efficacy.

WHO is not responsible, and does not accept any liability, for the testing of pesticides for compliance with the specifications, nor for any methods recommended and/or used for testing compliance. As a result, WHO does not in any way warrant or represent that any pesticide claimed to comply with a WHO specification actually does so.

¹ This disclaimer applies to all specifications published by WHO.

INTRODUCTION

WHO establishes and publishes specifications* for technical material and related formulations of public health pesticides with the objective that these specifications may be used to provide an international point of reference against which products can be judged either for regulatory purposes or in commercial dealings.

From 2002, the development of WHO specifications follows the **New Procedure**, described in the Manual for Development and Use of FAO and WHO Specifications for Pesticides. This **New Procedure** follows a formal and transparent evaluation process. It describes the minimum data package, the procedure and evaluation applied by WHO and the experts of the “FAO/WHO Joint Meeting on Pesticide Specifications” (JMPS).

WHO Specifications now only apply to products for which the technical materials have been evaluated. Consequently, from the year 2002 onwards the publication of WHO specifications under the **New Procedure** has changed. Every specification consists now of two parts, namely the specifications and the evaluation report(s):

Part One: The Specification of the technical material and the related formulations of the pesticide in accordance with chapters 4 to 9 of the above-mentioned manual.

Part Two: The Evaluation Report(s) of the pesticide, reflecting the evaluation of the data package carried out by WHO and the JMPS. The data are provided by the manufacturer(s) according to the requirements of chapter 3 of the above-mentioned manual and supported by other information sources. The Evaluation Report includes the name(s) of the manufacturer(s) whose technical material has been evaluated. Evaluation reports on specifications developed subsequently to the original set of specifications are added in a chronological order to this report.

WHO specifications under the **New Procedure** do not necessarily apply to nominally similar products of other manufacturer(s), nor to those where the active ingredient is produced by other routes of manufacture. WHO has the possibility to extend the scope of the specifications to similar products but only when the JMPS has been satisfied that the additional products are equivalent to that which formed the basis of the reference specification.

Specifications bear the date (month and year) of publication of the current version. Dates of publication of the earlier versions, if any, are identified in a footnote. Evaluations bear the date (year) of the meeting at which the recommendations were made by the JMPS.

* Footnote: The publications are available on the Internet under (<http://www.who.int/whopes/quality/en/>).

PART ONE
SPECIFICATIONS

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WHO SPECIFICATIONS FOR PUBLIC HEALTH PESTICIDES

***Bacillus thuringiensis subspecies israelensis* strain AM65-52**

INFORMATION

Scientific name

Bacillus thuringiensis subsp. israelensis strain AM65-52.

Shorthand terms

Bt: all subspecies of *Bacillus thuringiensis*.

Bti: all strains of *Bacillus thuringiensis subspecies israelensis* (flagella serotype H-14).

Bti AM65-52: the strain to which the CIPAC code 770 applies.

CIPAC number

770

Identity tests

- (i) Microscopic examination: gram-positive rods; presence of spores and parasporal crystalline inclusions.
- (ii) SDS-PAGE analysis of molecular weight profile of the endotoxin protein crystals.
- (iii) Agarose-GE analysis of the plasmid profile.

An alternate strain specific identity methodology may be used: *Genomotyping*.

Bacterial strains can be characterized by comparing their genomic DNA to an array of genomic DNA fragments originating from a mixture of different strains of the same species. Background information on the potential of the technology and its resolution is given in the following references (Salama et al. 2000, Leavis et al. 2007, Vlamincx et al. 2007). This current hybridization technology has permitted the assaying of thousands of nucleic acid sequences in a single reaction on a solid substrate. Such a massively parallel system offers the opportunity of diagnostic applications for strain identification through a comparative process.

Definition of active ingredient

A mixture of free endotoxin protein crystals produced by Bti AM65-52 and the spores and cells bearing them.

Measurement of active ingredient activity

Bioassay with 4th instar larvae of *Aedes aegypti* (strain Bora Bora), results expressed as international toxic units (ITU)/mg product, relative to a reference Bti material. Note: the only reference standard currently available is Valent BioSciences Corp. strain AM65-52, lot #82-691-W5, which has a biopotency of 7992 ITU/mg.

WHO SPECIFICATIONS FOR PUBLIC HEALTH PESTICIDES

***Bacillus thuringiensis* subspecies *israelensis*, strain AM65-52, WATER-DISPERSIBLE GRANULES**

WHO specification 770/WG (October 2012)

This specification, which is PART ONE of this publication, is based on an evaluation of data submitted by the manufacturer whose name is listed in the evaluation reports (770/2006, 770/2011). It should be applicable to relevant products of that manufacturer but it is not an endorsement of those products, nor a guarantee that they comply with the specification. The specification may not be appropriate for the products of other manufacturers. The evaluation reports (770/2006, 770/2011), as PART TWO, form an integral part of this publication.

1 Description (Note 1)

The material shall consist of a homogeneous mixture of *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52 (Notes 2 & 3), together with fillers and any other necessary formulants. It shall be in the form of small pale brown granules (Note 4), intended for spray application after disintegration and dispersion in water, or for direct application to mosquito larval habitats including water storage containers. The formulation shall be dry, free flowing, and free from visible extraneous matter and hard lumps.

2 Active ingredient (Note 1)

2.1 Identity

The active ingredient shall comply with the identity tests described in Note 5.

2.2. *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52, content (Note 6)

The *Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 biological activity (biopotency) shall not be less than 2700 International Toxic Units/mg, when determined by the method described in Note 6.

3 Relevant impurities (Notes 1 & 7)

3.1 Water (MT 30.5, CIPAC Handbook J, p.120, 2000)

Maximum: 50 g/kg.

4 Bacterial contaminants (Note 1)

4.1 *Staphylococcus aureus* (Note 8)

Staphylococcus aureus shall not be detected when tested by the method described in Note 8.

4.2 *Salmonella* species (Note 9)

Salmonella species shall not be detected when tested by the method described in Note 9.

4.3 ***Pseudomonas aeruginosa*** (Note 10)

Pseudomonas aeruginosa shall not be detected when tested by the method described in Note 10.

4.4 ***Escherichia coli*** (Note 11)

Escherichia coli shall not exceed 100 colony-forming units (CFU)/g of WG when tested by the method described in Note 11.

5 **Physical properties** (Note 1)

5.1 **pH range** (MT 75.3, CIPAC Handbook J, p.131, 2000)

pH range: 5.6 to 6.0.

5.2 **Persistent foam** (CIPAC MT 47.2, CIPAC Handbook F, p.152, 1995)

No measurable foam, immediately (Note 12).

5.3 **Wet sieve test** (MT 185, CIPAC Handbook K, p.149, 2003)

Not more than 2.2% of the formulation shall be retained on a 75 µm test sieve.

5.4 **Degree of dispersion** (MT 174, CIPAC Handbook F, p.435, 1995) (Note 13)

A minimum of 90% of the *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52, content found under 2.2 shall be in suspension after 5 min in CIPAC Standard Water D at $30 \pm 2^\circ\text{C}$.

5.5 **Suspensibility** (MT 184, CIPAC Handbook K, p.142, 2003) (Note 13)

A minimum of 90% of the *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52, content found under 2.2 shall be in suspension after 30 min in CIPAC Standard Water D at $30 \pm 2^\circ\text{C}$.

5.6 **Wettability** (MT 53.3, CIPAC Handbook F, p.164, 1995)

The formulation shall be completely wetted in 5 seconds, without swirling.

5.7 **Dustiness** (MT 171, CIPAC Handbook F, p.425, 1995) (Note 14)

Nearly dust-free.

6 **Storage stability**

6.1 **Stability at elevated temperature** (MT 46.3, CIPAC Handbook J, p.128, 2000) (Note 15)

After storage for 14 days at $54 \pm 2^\circ\text{C}$, the determined average active ingredient content shall not be lower than 84%, relative to the determined average found before storage (Note 16), and the formulation shall continue to comply with the clauses for:

- pH range (5.1);
- wet sieve test (5.3);
- degree of dispersion (5.4);
- suspensibility (5.5);
- dustiness (5.7).

Note 1 A sample consisting of at least two sealed bags (or the smallest packaging units) should be taken from each batch for testing. Prior to testing, sealed bags must not be opened and must be kept away from direct sunlight and other heat sources. The material to be tested for bacterial contaminants (clauses 4.1-4.5) must be taken from a bag freshly opened under aseptic conditions.

Note 2 Unlike most WHO specifications for formulations, a specification for the corresponding technical grade active ingredient is not cross-referenced in this case, because the WG is produced in an integrated process in which the active ingredient is not isolated.

Note 3 The active ingredient, Bti strain A65-52, is defined as a mixture of free endotoxin protein crystals and the Bti cells and spores bearing these endotoxin crystals.

Note 4 The granules have a musty odour.

Note 5 Identification of Bti strain AM65-52

Identification is based on the following tests.

- (i) Microscopic examination of the bacterial cells after gram staining (gram positive rods), and of spores and adherent crystalline proteins without gram staining.
- (ii) SDS-PAGE analysis of molecular weight profile of the Bti crystalline endotoxin proteins.
- (iii) Agarose gel electrophoresis of the plasmid DNA coding for the endotoxins.

In test (i), gram staining is a universally-used bacteriological test and is not described below. *Bacillus thuringiensis* is observed as gram-positive rods in test (i) but this result identifies only the broad group of bacteria which includes Bt. Microscopic observation of spores and adherent (irregularly round) crystals supports identification as Bt but is not definitive. Tests (ii) and (iii) identify the Bti strain as AM65-52. Identity may be established using either test (ii) or (iii) in combination with test (i) but, in cases of doubt, all tests should be conducted.

Flagellar antigens (H-14) may also be used to identify the presence of Bti, if suitable well-characterized antisera become available but it is important to note that such antisera would not identify the strain.

Identity test (ii), molecular weight profile of the Bti strain A65-52 endotoxin protein crystals

Principle

Endotoxins of Bti strain AM65-52 occur as irregularly round inclusions, developed during sporulation. The crystals contains 4 major proteins^{1,2}, designated Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. Crystals are extracted from the formulation by centrifugation and washing. The crystal proteins are dissolved and denatured (losing their secondary and tertiary structure) and molecular weights are determined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, based on that of Laemmli *et al.*³, as modified for Bt toxins by Brussock & Carrier⁴. Similarly-treated standard proteins are also separated on the gel, to provide molecular weight calibrants. After staining the gel and de-staining it to remove the background, 3 major protein bands should be apparent, of 135 kDa (Cry4Aa, Cry4Ba), 70 kDa (Cry11Aa), and 28 kDa (Cyt1Aa).

¹ Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**: 242-255.

² Crickmore *et al.* (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**: 807-813.

³ Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (5259): 680-685.

⁴ Brussock, S.M. and T.C. Carrier (1990) Use of sodium dodecyl sulfate – polyacrylamide gel electrophoresis to quantify *Bacillus thuringiensis* δ -endotoxins. Chapter in: *Analytical chemistry of Bacillus thuringiensis. ACS Symposium Series.* (Hickie, L.A. and W.L. Fitch. eds.) 78-87.

Equipment and materials

Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system; resolving gels of 10% acrylamide or a linear gradient of approximately 5-20% are appropriate.

Boiling water bath (100°C).

Micro-centrifuge (Eppendorf Microfuge, or equivalent), producing 8000 g.

Molecular weight calibration standard. Containing proteins in the range 14 kDa (lysozyme) to 200 kDa (myosin). Intermediate molecular weight proteins that may be included are β -galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and myoglobin (17 kDa). Examples of commercially available calibration kits are Mark 12 unstained standard (Invitrogen Cat.# LC5677) or Broad range SDS-PAGE standard (BioRad Cat.# 161-0317) but any suitable equivalent may be used. The calibration standard should be prepared in 2X Laemmli sample buffer.

Ethylenediamine tetra-acetic acid solution (EDTA), 5 mM in water, pH8.

Sodium chloride/EDTA solution, NaCl/EDTA, 1 M/5 mM in water, pH 8.

Sodium hydroxide solution, 0.1 M in water.

2X Laemmli buffer, 125 mM tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% β -mercaptoethanol, 50% glycerol, 0.02% bromophenol blue (tracking marker) in water. Dithiothreitol (0.2 M) may be used instead of β -mercaptoethanol.

Coomassie blue solution, 0.2% Coomassie Brilliant Blue R in water containing 50% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Methanol/acetic acid, water containing 25% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Deionised water.

Micropipette.

Method

- i. Weigh approximately 10 mg WG into a micro-centrifuge tube. Add NaCl/EDTA solution and disperse the product. Centrifuge at >8000 g until the suspended solids form a pellet (typically 5 min at 14000 g). Discard the supernatant.
- ii. Wash the pellet twice in 5 mM EDTA pH 8.0, centrifuge as above and discard the supernatant each time.
- iii. Solubilize the endotoxin crystals in the pellet by re-suspending them in 100 μ l NaOH solution for 30 min at 37°C.
- iv. Centrifuge the suspension, as above, to remove insoluble materials. Collect the supernatant and discard the pellet.
- v. Add 100 μ l 2X Laemmli buffer to the supernatant, mix and immediately heat the mixture at 100°C for 5 min.
- vi. Cool then centrifuge the mixture for 5 min at \geq 8000 g to remove insoluble materials. Collect the supernatant and discard the pellet.
- vii. Load a small volume (approximately 10-20 μ l) of supernatant onto an SDS-PAGE gel. Also load the gel with an appropriate quantity of molecular weight calibration standard in 2X Laemmli buffer. Perform the electrophoresis according to the gel rig manufacturer's instructions.
- viii. Stain the gel with Coomassie blue solution, to visualize the proteins, then de-stain it with methanol/acetic acid until the background is clear.
- ix. Observe the positions of the main distinct bands in the sample relative to the molecular weight calibration standard. Bti strain AM65-52 endotoxins are expected to produce bands at positions corresponding to approximately 135, 70 and 28 kDa.

Identity test (iii), agarose gel electrophoresis of the plasmid DNA

Principle

Bti spores are separated from the formulation, cultured in Luria broth, then lysed and centrifuged to remove insolubles. Following plasmid precipitation by low temperature ethanol and centrifugation, residual proteins and RNA are removed with proteinase and RNases, respectively. The plasmid DNA is separated by agarose gel electrophoresis and visualized using ethidium bromide fluorescence under UV light. Under these conditions, the Bti strain AM65-52 plasmid produces visible DNA bands corresponding to approximately 3.3, 4.2, 4.9, 10.6, 68, and 75 MDa, the last of which contains the Bti toxin genes. The 68 and 75 MDa components will generally appear as one band above the chromosomal smear. Additional plasmids known to be present are 105 and 135 MDa bands, which are too large to isolate easily.

Equipment and materials

Incubator, 37°C.

Water bath, 68°C.

Shaker water bath, 28°C.

Refrigerator, $4 \pm 2^\circ\text{C}$.

Freezer, $-18 \pm 2^\circ\text{C}$.

Ice, crushed.

Bench-top centrifuge, taking 50 ml tubes, to operate at 4000 g.

Micro-centrifuge, refrigerated (Eppendorf Microfuge, or equivalent), to operate at 14000 g.

Vortex mixer.

Vacuum dryer, Savant Speedvac or equivalent.

Luria broth, Sigma-Aldrich L3522 or equivalent, reconstituted according to the manufacturer's instructions and sterilized in an autoclave.

Water, double-distilled.

Hydrochloric acid, concentrated.

Acetic acid, glacial.

Tris buffer solution, 1 M. Dissolve 121.1 g tris base in about 800 ml water, adjust to pH 7.6 with concentrated HCl (about 60 ml) and make to 1 l with water.

Sodium chloride solution, 5 M. Dissolve 292.2 g NaCl in water and make to 1 l.

EDTA solution, 0.5 M in water, adjusted to pH 8.0.

Sodium dodecyl sulfate (SDS) solution, electrophoresis grade, 10% in water. Dissolve 100 g SDS in about 900 ml (heating to 68°C to assist dissolution), adjust to pH 7.2 with a few drops of concentrated HCl and make to 1 l with water.

TES buffer solution. Dilute a mixture of 3 ml tris buffer; 1 ml EDTA and 1 ml NaCl solutions (as above) to 100 ml with water.

Sucrose medium. Dilute 12.50 g sucrose together with 1 ml NaCl and 2.5 ml tris solutions to 50 ml with water.

SDS-NaCl solution. Dilute a mixture of 2 ml SDS and 1.4 ml NaCl solutions to 10 ml with water.

Sodium acetate solution. Dissolve 40.81 g sodium acetate $\cdot 3\text{H}_2\text{O}$ in about 80 ml water, adjust to pH 5.6 with glacial acetic acid and make to 100 ml with water.

Tris-borate buffer solution. Dissolve 108 g tris base, 55 g boric acid and 5 ml EDTA solution in about 800 ml water, adjust to pH 8.3 and dilute to 1 l (10X tris-borate buffer). Dilute 1+9 with water to produce 1X tris-borate buffer.

Lysozyme solution. 50 mg/ml in sucrose medium.

Ethanol, 100% and 70% aqueous solution, cooled to 4°C.

T1 RNase solution, 100 U/ml.

RNase A solution, 10 mg/ml.

Proteinase solution, 10 mg/ml.

Agarose gels. Prepare 0.8% gels in 1X tris-borate buffer. A gel 20cm long, 10cm wide and 3-4mm deep requires about 100 ml agarose solution. Use 1.5% agar for end plugs, if required. When the gel has solidified, cover its surface minimally with tris-borate buffer (approximately 40 ml).

PVDC film wrap ("cling-film"), Saran™ or equivalent.

Electrophoresis apparatus, suitable for running agarose gels. BioRad; GE (formerly Pharmacia) or equivalent.

DNA molecular weight marker solution, 1kB ladder (Invitrogen), Pulse marker (Sigma) or equivalent.

Tracking dye for electrophoresis, containing 0.25% bromophenol blue and 15% Ficoll 400.

Ethidium bromide solution, 5 µg/ml in water (note: wear nitrile gloves for handling solution and treated gels).

UV lamp, for visualization of DNA bands.

Method

- i. Aseptically transfer about 1 mg of Bti WG into 2 ml medium/water in a sterile bottle and mix thoroughly.
- ii. Maintaining aseptic conditions, inoculate 100 µl of the suspended Bti cell mixture into 20 ml Luria broth and incubate, with shaking, at 28°C for about 16 hours.
- iii. Sediment the cells in a bench-top centrifuge at maximum speed for 15 minutes and discard the supernatant.
- iv. Add 1 ml TES buffer, vortex to re-suspend the pellet, transfer the suspension to a micro-centrifuge tube and sediment the cells for 2 minutes at 5°C. Discard the supernatant.
- v. Add 180 µl sucrose medium and vortex to re-suspend the pellet. Add 20 µl lysozyme solution, mix gently by hand (do not use a vortex mixer) and incubate at 37°C for 60 minutes.
- vi. Add 48 µl NaCl solution, 12 µl EDTA solution and 260 µl SDS-NaCl solution and slowly invert the tube, twice. Incubate the mixture for 10 minutes at 68°C, then stand the tube in ice for 60 minutes. Centrifuge at 4°C for 15 minutes to sediment the cell wall debris and transfer 300 µl of supernatant to another micro-centrifuge tube.
- vii. Add 33 µl sodium acetate solution and 670 µl cold 100% ethanol, vortex to mix and place in the freezer for ≥1 hour. Centrifuge at 5°C for 15 minutes and discard the supernatant.
- viii. Add approximately 200 µl cold 70% ethanol, vortex to mix, then centrifuge at 5°C for 10 minutes and discard the supernatant. Dry the pellet in a vacuum dryer for about 30 minutes. Add 200 µl TES buffer to the dried pellet, vortex to re-suspend it, allow the mixture to stand at room temperature for 15 minutes and then vortex again to mix.
- ix. Add 2 µl T1 RNase solution and 2 µl RNase A solution, mix and incubate the mixture at 37°C for 30 minutes. Add 20 µl proteinase solution, mix and incubate the mixture at 37°C for 1 hour.
- x. Mix 15 µl sample solution with 3 µl tracking dye and transfer the whole to a well in the agarose gel. Include an appropriate amount of DNA molecular weight marker solution, according to the manufacturer's directions, in an adjacent well.
- xi. Run the gel at 50 V for about 15 minutes. Turn off the voltage before removing excess buffer from the surface of the gel and then cover it with PVDC film. Adjust the voltage to give 20 mA current and run the gel overnight (16-17 hours). Reverse the voltage polarity for 30 seconds immediately before switching off and removing the gel.
- xii. Stain the gel in ethidium bromide solution for 20 minutes, with gentle rocking. Destain the gel in 1X tris-borate buffer for 20 minutes, changing the buffer 3 times during this time. Place the gel under a UV lamp and photograph it. Bti strain AM65-52 plasmid should produce 5 fluorescent bands below the chromosomal smear and, depending upon the separation quality of the gel, 1 band may appear above the chromosomal smear. Due to possible conformational changes from super-coiled to relaxed forms of the plasmid during preparation, the actual sizes of the plasmids are best determined by comparison on the same gel with a Bt strain having known plasmid sizes, such as Bti reference standards HD-1 or HD-2.

Note 6 **Determination of biopotency**

Principle

Biopotency is measured in International Toxic Units (ITU) per mg of product. Biopotency is tested by comparing mosquito larval mortality produced by the product under test with the mortality produced by a spray-dried reference powder* of *Bacillus thuringiensis* subsp. *israelensis*, using early fourth-instar larvae of *Aedes aegypti* (strain Bora Bora). The toxicity (ITU/mg) of products tested is determined according to the following formula:

$$\text{ITU/mg of product tested} = \frac{\text{reference standard ITU/mg} \times \text{LC}_{50} \text{ (mg/l) standard}}{\text{LC}_{50} \text{ (mg/l) product tested}}$$

Equipment and materials

Top-drive homogenizer or stirrer

Ice bath (container of crushed ice)

Analytical balance (accurate to ± 0.1 mg)

Top-pan balance (accurate to ± 10 mg), preferably with tare facility

Deionised water

Wetting agent (e.g. Tween 80)

200 ml beakers, borosilicate glass or plastic

500 ml bottle, wide-necked, screw-capped, clear glass

100 ml bottles, screw-capped clear glass

Micropipette

10 ml pipette

12 ml tubes, plastic with stoppers or caps

200 ml cups, plastic or wax-coated paper

Method

(i) Production of test larvae

L4 larvae are representative of the total sensitivity of the target population and convenient to handle. It is very important to use a homogenous population of early fourth instars, which are obtained within five days of hatching, using standardized rearing methods.

Aedes aegypti eggs are laid in a cup lined with filter paper and one-third filled with deionised water. The paper is dried at room temperature and kept for several months by storing in a sealed plastic bag at room temperature. When larvae are needed, the paper is immersed in de-chlorinated water. To synchronise hatching, add larval feed to the water 24 hours prior to adding the eggs. The bacterial growth will deoxygenate the water and this triggers egg hatching. This usually induces the first instars to hatch within 12 h. These larvae are then transferred to a container (25 x 25 x 10 cm) containing 2 litres of de-chlorinated water, to obtain a population of 500 to 700 larvae per container. Larval feed may be flakes of protein as used for aquarium fish, or powdered cat biscuit, and the containers are held at $25 \pm 2^\circ\text{C}$. It is important that the amount of food is kept low to avoid strong bacterial growth that kills the larvae. Several feeds, at 1- to 2-day intervals and daily observation of the larvae is optimal. If the water becomes turbid, replace all water by filtering out the larvae and transfer to a clean container with clean water and feed. Five to seven days later, an homogeneous population of early fourth instars (5 days old and 4 to 5 mm in length) should be obtained.

* The original reference powder recommended by WHO for this purpose, IPS82 strain 1884 from Pasteur Institute, is no longer available. Until a replacement international reference powder of Bti becomes available, a reference standard of strain AM65-52 may be obtained from Valent Biosciences Corp. for the purposes of testing product compliance with the specification. This reference standard of strain AM65-52 was calibrated against IPS82 strain 1884 and has a biopotency of 7992 ITU/mg.

(ii) Preparation of reference standard suspensions for calibration of the bioassay

Before preparing the suspension, check that stirring/blending of the wetting agent/water mixture, described in the following paragraph, does not lead to foaming. If it does, dilute (e.g. 1:10) the wetting agent before use.

Accurately weigh about 50 mg (to the nearest 0.1 mg) of the reference standard powder and transfer it to a 200 ml beaker with 100 ml deionised water (it can be transferred directly to the 500 ml bottle if the neck is wide enough to accept the stirrer/blender head). Allow the mixture to stand for 30 min and add a small drop (about 0.2 mg) of wetting agent. Place the beaker in the ice bath and either stir or blend the mixture for 2 min. Check visually for any large particulates remaining and repeat the stirring/blending if there are any. Weigh or tare the 500 ml bottle and transfer the suspension/solution to it, rinsing carefully and thoroughly the beaker and stirrer/blender. Add further deionised water to make the weight of contents to 500 g (500 ml), cap the bottle and shake vigorously to mix the contents. Confirm, by microscopic examination of a small aliquot, that no aggregates of spores and crystals persist. If any are present, the contents must be subjected to further stirring/blending in the ice bath. This primary suspension/solution contains 1 mg/10 ml and must be shaken vigorously immediately before removing aliquots.

Transfer 10 ml aliquots of the primary solution/suspension to clean 12 ml tubes that are stoppered/capped immediately. If transferring a number of aliquots, cap and shake the primary suspension/solution at intervals not exceeding 3 min, because the spores and crystals settle quickly in water. The aliquots can be stored for a month at 4°C and for 2 years in a freezer at -18°C. Each contains 1 mg standard powder.

To prepare a “stock solution”, weigh or tare a 100 ml bottle. Transfer one of the 10 ml aliquots into the 100 ml bottle, rinsing carefully at least twice with deionised water, and fill to a total of 100 g. Shake the mixture vigorously (or use the blender) to produce an homogeneous suspension. Frozen aliquots must be homogenised thoroughly before use, because particles agglomerate during freezing. The “stock solution” contains 10 mg/l.

From the “stock solution”, subsequent dilutions are prepared directly in plastic cups filled (by weighing) with 150 ml de-ionized water. To each cup, 25 early L4 larvae of *Aedes aegypti* are added first by means of a Pasteur pipette, prior to addition of bacterial suspensions. The volume of water added with the larvae is removed from the cup (by weighing) and discarded, to avoid changing of the volume of liquid in the cup. Using micropipettes, 600 µl, 450 µl, 300 µl, 150 µl, 120 µl and 75 µl of “stock solution” are added to separate cups and the solutions mixed to produce final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l, respectively, of the reference standard powder. Four replicate cups are used for each concentration and one for the control, which contains only 150 ml de-ionized water.

(iii) Preparation of suspensions of the product to be tested

An initial homogenate is made in the same manner as described for the reference standard powder, above, except that the replicate determinations must be made on dilutions prepared by weighing separate test portions of the product. That is four replicate primary suspension/solutions must be prepared. Cups and larvae are prepared as described above and comparable dilutions are prepared as for the reference standard.

(iv) Determination of toxicity

No food is added for *Aedes* larvae. All tests should be conducted at $28 \pm 2^\circ\text{C}$, with a 12 h light, 12 h dark cycle. To avoid the adverse effects of evaporation of water in low humidity, the relative humidity should be maintained at $50 \pm 15\%$, if possible.

Each bioassay series should preferably involve 6 concentrations x 4 replicates x 25 larvae for the reference standard and the unknown and 100 larvae for the control. The aim is to identify a range of concentrations that give mortality between 5-95% (because 100 larvae are used). Data giving 0 or 100% mortality are ignored for the calculation of the LC_{50} . To prepare a valid dose-response curve, only concentrations giving between 95 and 5% mortality should be used. Within this range, a minimum of two concentrations must be above the LC_{50} and two below, to ensure the validity of the LC_{50} value (the sensitivity of the insect colony may require a slightly different 6-dilution series to be used).

Mortality is determined at 24 and 48 h by counting the live larvae remaining. If pupation occurs, the pupae should be removed and their numbers excluded from the calculations. If more than 5% of larvae pupate, the test is invalidated because larvae do not ingest 24 hours before pupation and too many larvae may have survived simply because they were too old. Because of the very rapid killing action of Bti, usually there is no difference between the 24 and 48 h mortality. In this case, the 48-h count confirms the 24-h reading and provides a check on the possible influence of factors other than Bti components.

If the control mortality exceeds 5%, the mortalities of treated groups should be corrected according to Abbott's formula [Abbott, W. S., (1925). A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology*, **18**, 265-267]:

$$\text{percentage (\%) control} = \frac{X - Y}{X \times 100}$$

where: X = % survival in untreated control;
Y = % survival in treated sample.

Tests with a control mortality greater than 10%, or any pupation greater than 5%, should be discarded. Mortality-concentration regression lines may be drawn on gauss-logarithmic paper but this is rather subjective. It is preferable to use a statistical program, such as SAS, which incorporates Log Probit Analysis. With such a statistical program, Abbott's formula is not required because the correction is automatically carried out by the program. The toxicity is determined by estimation and comparison of the LC₅₀s of the tested product and reference standard preparations, using the formula described above. The toxicity is defined by the count at 24 h after initiation of the test.

For increased accuracy, bioassays should be repeated on at least three different days, concurrently with the assay of the reference standard, and the standard deviation of the means calculated. A test series is valid if the relative standard deviation (RSD) is <25%.

Note 7 Beta-exotoxin (a heat-stable nucleotide composed of adenine, glucose and allaric acid) has been shown not to occur in the products of the manufacturer identified in evaluation report 770/2006 and its presence is unlikely to occur spontaneously. However, beta-exotoxin can be generated by some strains of *Bacillus thuringiensis* and, if detectable by the method of Bond *et al.**, it would be designated as a relevant impurity and a clause would be required to limit its concentration.

Note 8 ***Staphylococcus aureus* Enumeration and Identification**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%

Incubator, controlled to within the range 35-37°C

Sterile phosphate buffer, pH 7.2 (USP or equivalent)

Sterile, pre-poured Baird-Parker agar plates (Difco 0768-01-1, BBL 11023, or equivalent), supplemented with *egg yolk tellurite enrichment* (Difco 0779-73-1 or equivalent)

Mammalian plasma (rabbit coagulase plasma, lyophilized, BBL 40658 or equivalent)

Sterile bottles, capped, at least 100 ml

Sterile test tubes

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml sterile phosphate buffer. Mix well to disintegrate and disperse the formulation.

In duplicate, transfer 1 ml suspension (0.1 g WG) onto the surface of agar plates. Spread the inoculum evenly across the surface and allow it to sink into the surface of the agar. Cover, invert and incubate the plates for 21-26 hours before examining them.

* Bond R.P.M., *et al.* The thermostable exotoxin of *Bacillus thuringiensis*. In: Burges H. D. and Hussey N. W., eds. *Microbial control of insects and mites*. Academic Press, London, 1971.

S. aureus forms black, shiny, convex colonies surrounded by a clear zone on Baird-Parker agar. If negative for *Staphylococcus*, incubate the plates for an additional 24 hours and read again. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. Perform gram stains on a typical suspect colony from each plate. *S. aureus* are gram positive cocci occurring in clusters. If the cells do not conform to this description, *S. aureus* can be considered absent from the sample. However, if the cells conform to the description, perform the following test.
- ii. Coagulase test. Using a sterile inoculating loop, transfer a portion of a typical suspect colony to 0.5 ml mammalian plasma in a sterile test tube, and swirl to mix. Conduct the test in parallel with positive and negative controls. Place the tubes in the incubator, examine them after 3 hours and then at suitable intervals for a total of 24 hours. The positive and negative controls should show coagulation and no coagulation, respectively. If the test of the suspect colony shows no visible coagulation, it can be concluded that coagulase positive *S. aureus* is absent.

Note 9

Salmonella species Detection

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$

Incubator, controlled to within the range 35-37°C

Lactose broth, Difco 0004, BBL 11333, or equivalent

Selenite-cystine broth, 10 ml capped test tubes (Difco 0687, BBL 11606, or equivalent)

Sterile, pre-poured brilliant green agar plates, (Difco 0014, BBL 11073, or equivalent)

Sterile, pre-poured xylose-lysine-desoxycholate (XLD) agar plates, (Difco 0788, BBL 11838, or equivalent)

Sterile, pre-poured bismuth sulfite agar plates, (Difco 0073, BBL 11031, or equivalent)

Sterile, pre-prepared butt-slant tube containing approximately 10 ml triple sugar iron agar (Difco 0265, BBL 11749, or equivalent)

Brilliant green solution, USP (1:1000 aqueous solution prepared and stored at 2-8°C)

Iodine-iodide solution, USP (Dissolve 5 g potassium iodide and 6 g iodine in 20 ml USP purified water; store at 2-8°C)

Fluid tetrathionate broth (Difco 0104, BBL 11705, or commercially-prepared equivalent), 10 ml in capped test tubes. To each 10 ml tetrathionate broth tube, add 0.1 ml of the prepared brilliant green solution; mix, then add 0.2 ml prepared iodine-iodide solution. Mix.

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml sterile lactose broth. Mix well to disintegrate and disperse the formulation and incubate for 24 hours.

Transfer 1 ml portions (0.1 g WG) of the incubated culture into two separate tubes containing, respectively, 10 ml selenite cystine broth and 10 ml fluid tetrathionate broth containing iodide-iodine solution and brilliant green solution. Mix and incubate the inoculated tubes for 18-24 hours.

Using an inoculating loop, streak portions of the incubated selenite cystine and tetrathionate tubes onto separate plates of brilliant green agar, XLD agar, and bismuth sulfite agar. Cover, invert and incubate the plates for 18-24 hours, or for up to 48 hours in the case of bismuth sulfite agar, before examining them.

Salmonella colonies exhibit the following characteristics.

Brilliant green agar – small transparent colourless, or pink/white opaque, colonies, later becoming surrounded by a pink to red zone.

XLD agar – red colonies, with or without black centres.

Bismuth sulfite agar – black or dark green colonies.

If none of the colonies match these descriptions, the sample meets the requirement for absence of *Salmonella* species. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. If colonies matching any of the above descriptions are found, perform a gram stain on material taken from them. *Salmonella* species are gram negative rods. If the cells match this description, proceed with confirmatory test ii, below.
- ii. With an inoculating needle or loop, transfer material from the suspect colonies to a butt-slant tube containing triple sugar iron (TSI) agar. First stab the needle/loop beneath the surface, then streak the slant. Incubate the tube(s) for 12-24 hours. *Salmonella* species typically ferment glucose with the production of acid and some species also produce gas and H₂S (Table 1).

The tube, if positive for *Salmonella*, will have an alkaline (red) slant and an acid (yellow) butt with or without blackening of the butt from H₂S production. If the presence of *Salmonella* is indicated, proceed further to identify the organism by employing the Vitek/Vitek2 automated microbial identification system, other approved identification system, or by performing appropriate biochemical and cultural reactions.

Table 1. Reactions observed in TSI agar

Reaction	Explanation
Acid butt (yellow), alkaline slant (red)	Glucose fermented
Acid throughout medium, butt and slant yellow	Lactose or sucrose, or both, fermented
Gas bubble in butt, medium sometimes split	Aerogenic culture
Blackening in the butt	Hydrogen sulfide produced
Alkaline slant and butt (medium entirely red)	None of the three sugars fermented

Note 10 *Pseudomonas aeruginosa* Enumeration and Identification

Equipment and materials

Balance, with tare, capable of weighing 10 g to within ±1%.

Incubator, controlled to within the range 35-37°C.

Filter papers.

Sterile phosphate buffer, pH 7.2 (USP or equivalent)

Sterile, pre-poured cetrimide agar plates, (BBL 11554-pseudoseal agar, or equivalent).

N,N-dimethyl-p-phenylenediamine dihydrochloride.

Oxidase reagent (DrySlide® BBL 231746, or equivalent).

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml sterile USP phosphate buffer. Mix well to disintegrate and disperse the formulation.

Transfer, in duplicate, 1 ml portions (0.1 g WG) of the suspension onto the surface of cetrimide agar plates. Spread the inoculum evenly across the surface of the plates, cover and allow the inoculum to sink into the surface. Invert and incubate the plates for 48-72 hours before examining them.

P. aeruginosa forms characteristic bluish or greenish colonies. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. Perform a gram stain. *P. aeruginosa* cells are slender, gram negative rods.
- ii. Perform an oxidase test.

Either Using a platinum wire loop or sterile wooden stick, transfer a portion of the suspect colony to a DrySlide® oxidase reaction area. Spread the inoculum on

the reaction area to a 3-4 mm size. Examine the reaction area within 20 seconds. Positive reaction: organisms produce a purple to dark colour within 20 sec. Negative reaction: organisms produce no colour change, or a change to light grey, within 20 sec.

Or Use filter paper impregnated with either *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride or wetted with a drop of oxidase reagent. Concurrently perform the test on a *P. aeruginosa* reference culture, as a positive control. If a purple colour does not develop within 30 seconds, the result is negative.

- iii. If necessary, additional confirmation may be sought using biochemical or cultural tests appropriate for the identification of oxidase-positive, gram-negative, non-fermenting rods.

Note 11 *Escherichia coli* Enumeration (pour plate method)

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$.

Incubator, controlled to within the range 35-37°C.

Long-wavelength UV light source.

Sterile phosphate buffer, pH 7.2 (USP or equivalent).

Violet red bile agar with 4-methylumbelliferyl- β -D-glucuronide (VRB agar with MUG) (Difco 229100 or equivalent).

Sterile Petri dishes.

Method

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml sterile phosphate buffer. Mix well to disintegrate and disperse the formulation. If necessary, dilute further with sterile phosphate buffer, with thorough mixing, so that 1 ml will yield not more than 300 colonies.

In duplicate, transfer 1 ml suspension (0.1 g WG if the suspension has not been further diluted) into each of two sterile Petri dishes. Add to each dish approximately 15-20 ml of VRB agar with MUG, that has been cooled to about 45°C.

Cover the Petri dishes, mix the suspension with the agar by rotating the dishes in one direction and then in the opposite direction. Allow the contents to solidify at room temperature, invert the plates and incubate at 35-37°C for 22-26 hours.

Examine the plates for growth and, using the UV light source, for fluorescent colonies. Typical strains of *E. coli* (red colonies surrounded by a bile precipitate) exhibit a bluish fluorescent halo (MUG-positive). Apparent positives should be subjected to confirmatory tests. If confirmed as *E. coli*, count the number of MUG-positive colonies and calculate the average count for the two plates. Do not count colonies of non-*E. coli* coliforms, which may also produce red colonies with zones of precipitated bile but are MUG-negative.

Confirmatory tests

- i. Presumptive colonies of *E. coli* should be confirmed using either a Vitek automated microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *E. coli*.

Note 12 The test should be conducted with the formulation at 0.67 g/ml in water, which exceeds the highest rate of use recommended by the manufacturer. The test is to be conducted in CIPAC standard water D.

Note 13 Bioassay is the only fully reliable method to measure the content (biopotency) of active ingredient still in suspension. However, simpler methods such as gravimetric determination may be used on a routine basis provided that these methods have been shown to give equal results to those of the bioassay method. In case of dispute, the bioassay method shall be the referee method.

Note 14 Measurement of dustiness must be carried out on the sample "as received" and, where practicable, the sample should be taken from a newly opened container, because changes

in the water content of samples may influence dustiness significantly. The optical method, MT 171.2, usually shows good correlation with the gravimetric method, MT 171.1, and can, therefore, be used as an alternative where the equipment is available. Where the correlation is in doubt, it must be checked with the formulation to be tested. In case of dispute the gravimetric method shall be used.

Note 15 Tests for bacterial contaminants (clauses 4.1-4.4) are not specified after storage of the product for 14 days at 54°C, because this regime is unlikely to reveal the extent of potential proliferation that might occur under normal storage conditions.

Note 16 Samples representing “before” and “after” the storage stability test should be tested concurrently after the test, in order to minimize the variation occurring in assays of the biopotency. Material for the “before” test sample should be stored in a sealed container at 2-8°C, for the duration of the test, prior to bioassay. If the container is stored for this purpose in a refrigerator or freezer, it must be equilibrated to room temperature and dried externally before opening, to avoid contaminating the granules with atmospheric moisture which could affect the results of tests such as biopotency and dustiness.

WHO SPECIFICATIONS FOR PUBLIC HEALTH PESTICIDES

***Bacillus thuringiensis* subspecies *israelensis*, strain AM65-52, GRANULES**

WHO specification 770/GR (October 2012)

This specification, which is PART ONE of this publication, is based on an evaluation of data submitted by the manufacturer whose name is listed in the evaluation report (770/2011). It should be applicable to relevant products of that manufacturer but it is not an endorsement of those products, nor a guarantee that they comply with the specification. The specification may not be appropriate for the products of other manufacturers. The evaluation report (770/2011), as PART TWO, form an integral part of this publication.

1 Description (Note 1)

The material shall consist of granules containing *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52 (Note 2), together with suitable carriers and any other necessary formulants. It shall be in the form of small granules with a narrow particle size distribution (Note 3), intended for direct application to mosquito larval habitats. The formulation shall be dry, free from visible extraneous matter and hard lumps, free-flowing, essentially non-dusty and intended for application by machine or hand.

2 Active ingredient (Note 1)

2.1 Identity

The active ingredient shall comply with the identity tests described in Note 4.

2.2. *Bacillus thuringiensis* ssp. *israelensis*, strain AM65-52, content (Note 5)

The *Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 biological activity (biopotency) shall not be less than 200 International Toxic Units/mg, when determined by the method described in Note 5.

3 Relevant impurities (Notes 1 & 6)

3.1 Water (MT 30.5, CIPAC Handbook J, p.120, 2000)

Maximum: 30 g/kg.

4 Bacterial contaminants (Note 1)

4.1 *Staphylococcus aureus* (Note 7)

Staphylococcus aureus shall not be detected when tested by the method described in Note 7.

4.2 *Salmonella* species (Note 8)

Salmonella species shall not be detected when tested by the method described in Note 8.

4.3 ***Pseudomonas aeruginosa*** (Note 9)

Pseudomonas aeruginosa shall not be detected when tested by the method described in Note 9.

4.4 ***Escherichia coli*** (Note 10)

Escherichia coli shall not exceed 100 colony-forming units (CFU)/g of GR when tested by the method described in Note 10.

5 **Physical properties** (Note 1)

5.1 **pH range** (MT 75.3, CIPAC Handbook J, p.131, 2000)

pH range: 4.5 to 7.0.

5.2 **Pour and tap density** (MT 186, CIPAC Handbook K, p.151, 2003)

Pour density: 0.6 to 0.7 g/ml.

Tap density: 0.7 to 0.8 g/ml.

5.3 **Nominal size range** (MT 170, CIPAC Handbook F, p.420, 1995)

Not less than 900 g/kg of the formulation shall be within the size range of 841 to 2000 µm.

5.4 **Dustiness** (MT 171, CIPAC Handbook F, p.425, 1995) (Note 11)

Nearly dust-free.

5.5 **Attrition resistance** (MT 178, CIPAC Handbook H, p.304, 1998)

Minimum 97% attrition resistance.

6 **Storage stability**

6.1 **Stability at elevated temperature** (MT 46.3, CIPAC Handbook J, p.128, 2000) (Note 12)

After storage for 14 days at $54 \pm 2^{\circ}\text{C}$, the determined average biopotency shall not be lower than 70%, relative to the determined average found before storage (Note 13), and the formulation shall continue to comply with the clauses for:

- pH range (5.1);
- nominal size range (5.3);
- dustiness (5.4);
- attrition resistance (5.6).

Note 1 A sample consisting of at least two sealed bags (or the smallest packaging units) should be taken from each batch for testing. Prior to testing, sealed bags must not be opened and must be kept away from direct sunlight and other heat sources. The material to be tested for bacterial contaminants (clauses 4.1 - 4.4) must be taken from a bag freshly opened under aseptic conditions.

Note 2 The active ingredient, Bti strain AM65-52, is defined as a mixture of free endotoxin protein crystals and the Bti cells and spores bearing these endotoxin crystals.

Note 3 The granules have a musty odour.

Note 4 **Identification of Bti strain AM65-52**

Identification is based on the following tests.

- (i) Microscopic examination of the bacterial cells after gram staining (gram positive rods), and of spores and adherent crystalline proteins without gram staining.
- (ii) SDS-PAGE analysis of molecular weight profile of the Bti crystalline endotoxin proteins.
- (iii) Agarose gel electrophoresis of the plasmid DNA coding for the endotoxins.

In test (i), gram staining is a universally-used bacteriological test and is not described below. *Bacillus thuringiensis* is observed as gram-positive rods in test (i) but this result identifies only the broad group of bacteria which includes Bt. Microscopic observation of spores and adherent (irregularly round) crystals supports identification as Bt but is not definitive. Tests (ii) and (iii) identify the Bti strain as AM65-52. Identity may be established using either test (ii) or (iii) in combination with test (i) but, in cases of doubt, all tests should be conducted.

Flagellar antigens (H-14) may also be used to identify the presence of Bti, if suitable well characterized antisera become available but it is important to note that such antisera would not identify the strain.

Identity test (ii), molecular weight profile of the Bti strain A65-52 endotoxin protein crystals

Principle

Endotoxins of Bti strain AM65-52 occur as irregularly round inclusions, developed during sporulation. The crystals contains 4 major proteins^{1,2}, designated Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. Crystals are extracted from the formulation by agitation, centrifugation and washing. The crystal proteins are dissolved and denatured (losing their secondary and tertiary structure) and molecular weights are determined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, based on that of Laemmli *et al.*³, as modified for Bt toxins by Brussock & Carrier⁴. Similarly-treated standard proteins are also separated on the gel, to provide molecular weight calibrants. After staining the gel and de-staining it to remove the background, 3 major protein bands should be apparent, of 135 kDa (Cry4Aa, Cry4Ba), 70 kDa (Cry11Aa), and 28 kDa (Cyt1Aa).

Equipment and materials

Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system; resolving gels of 10% acrylamide or a linear gradient of approximately 5-20% are appropriate.

Boiling water bath (100°C).

Micro-centrifuge (Eppendorf Microfuge, or equivalent), producing 8000 g.

Molecular weight calibration standard. Containing proteins in the range 14 kDa (lysozyme) to 200 kDa (myosin). Intermediate molecular weight proteins that may be included are β -galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and myoglobin (17 kDa). Examples of commercially available calibration kits are Mark 12 unstained standard (Invitrogen Cat.# LC5677) or Broad range SDS-PAGE standard (BioRad Cat.# 161-0317) but any suitable equivalent may be used. The calibration standard should be prepared in 2X Laemmli sample buffer.

¹ Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**: 242-255.

² Crickmore *et al.* (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**: 807-813.

³ Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** (5259): 680-685.

⁴ Brussock, S.M. and T.C. Carrier (1990) Use of sodium dodecyl sulfate – polyacrylamide gel electrophoresis to quantify *Bacillus thuringiensis* δ -endotoxins. Chapter in: *Analytical chemistry of Bacillus thuringiensis*. ACS Symposium Series. (Hickie, L.A. and W.L. Fitch. eds.) 78-87.

Ethylenediamine tetra-acetic acid solution (EDTA), 5 mM in water, pH8.

Sodium chloride/EDTA solution, NaCl/EDTA, 1 M/5 mM in water, pH 8.

Sodium hydroxide solution, 0.1 M in water.

2X Laemmli buffer, 125 mM tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.2% β -mercaptoethanol, 50% glycerol, 0.02% bromophenol blue (tracking marker) in water. Dithiothreitol (0.2 M) may be used instead of β -mercaptoethanol.

Coomassie blue solution, 0.2% Coomassie Brilliant Blue R in water containing 50% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Methanol/acetic acid, water containing 25% methanol and 10% glacial acetic acid (or use a commercially available Coomassie-based staining system).

Deionised water.

Micropipette.

Method

- i. Weigh approximately 1 g Bti GR into a 50 ml screw cap tube. Add 3 ml of 0.2% Tween 80 and agitate granules in the solution by shaking for 30 minutes.
- ii. Aliquot 200 μ l of suspension, avoiding granules, into a clean microfuge tube.
- iii. Add 1 ml of NaCl/EDTA solution and disperse the product. Centrifuge at > 8000 g until the suspended solids form a pellet (typically 5 min at 14000 g). Discard the supernatant.
- iv. Wash the pellet twice in 5 mM EDTA pH 8.0, centrifuge as above and discard the supernatant each time.
- v. Solubilize the endotoxin crystals in the pellet by re-suspending them in 100 μ l NaOH solution for 30 min at 37°C.
- vi. Centrifuge the suspension, as above, to remove insoluble materials. Collect the supernatant and discard the pellet.
- vii. Add 100 μ l 2X Laemmli buffer to the supernatant, mix and immediately heat the mixture at 100°C for 5 min.
- viii. Cool, then centrifuge the mixture for 5 min at ≥ 8000 g to remove insoluble materials. Collect the supernatant and discard the pellet.
- ix. Load a small volume (approximately 10-20 μ l) of supernatant onto an SDS-PAGE gel. Also load the gel with an appropriate quantity of molecular weight calibration standard in 2X Laemmli buffer. Perform the electrophoresis according to the gel rig manufacturer's instructions.
- x. Stain the gel with Coomassie blue solution, to visualize the proteins, then de-stain it with methanol/acetic acid until the background is clear.
- xi. Observe the positions of the main distinct bands in the sample relative to the molecular weight calibration standard. Bti strain AM65-52 endotoxins are expected to produce bands at positions corresponding to approximately 135, 70 and 28 kDa.

Identity test (iii), agarose gel electrophoresis of the plasmid DNA

Principle

Bti spores are separated from the formulation, cultured in Luria broth, then lysed and centrifuged to remove insolubles. Following plasmid precipitation by low temperature ethanol and centrifugation, residual proteins and RNA are removed with proteinase and RNases, respectively. The plasmid DNA is separated by agarose gel electrophoresis and visualized using ethidium bromide fluorescence under UV light. Under these conditions, the Bti strain AM65-52 plasmid produces visible DNA bands corresponding to approximately 3.3, 4.2, 4.9, 10.6, 68, and 75 MDa, the last of which contains the Bti toxin genes. The 68 and 75 MDa components will generally appear as one band above the chromosomal smear. Additional plasmids known to be present are 105 and 135 MDa bands, which are too large to isolate easily.

Equipment and materials

Incubator, 37°C.

Water bath, 68°C.

Shaker water bath, 28°C.

Refrigerator, $4 \pm 2^\circ\text{C}$.

Freezer, -18 ± 2°C.

Ice, crushed.

Bench-top centrifuge, taking 50 ml tubes, to operate at 4000 g.

Micro-centrifuge, refrigerated (Eppendorf Microfuge, or equivalent), to operate at 14000 g.

Vortex mixer.

Vacuum dryer, Savant Speedvac or equivalent.

Luria broth, Sigma-Aldrich L3522 or equivalent, reconstituted according to the manufacturer's instructions and sterilized in an autoclave.

Water, double-distilled.

Hydrochloric acid, concentrated.

Acetic acid, glacial.

Tris buffer solution, 1 M. Dissolve 121.1 g tris base in about 800 ml water, adjust to pH 7.6 with concentrated HCl (about 60 ml) and make to 1 l with water.

Sodium chloride solution, 5 M. Dissolve 292.2 g NaCl in water and make to 1 l.

EDTA solution, 0.5 M in water, adjusted to pH 8.0.

Sodium dodecyl sulfate (SDS) solution, electrophoresis grade, 10% in water. Dissolve 100 g SDS in about 900 ml (heating to 68°C to assist dissolution), adjust to pH 7.2 with a few drops of concentrated HCl and make to 1 l with water.

TES buffer solution. Dilute a mixture of 3 ml tris buffer; 1 ml EDTA and 1 ml NaCl solutions (as above) to 100 ml with water.

Sucrose medium. Dilute 12.50 g sucrose together with 1 ml NaCl and 2.5 ml tris solutions to 50 ml with water.

SDS-NaCl solution. Dilute a mixture of 2 ml SDS and 1.4 ml NaCl solutions to 10 ml with water.

Sodium acetate solution. Dissolve 40.81 g sodium acetate·3H₂O in about 80 ml water, adjust to pH 5.6 with glacial acetic acid and make to 100 ml with water.

Tris-borate buffer solution. Dissolve 108 g tris base, 55 g boric acid and 5 ml EDTA solution in about 800 ml water, adjust to pH 8.3 and dilute to 1 l (10X tris-borate buffer). Dilute 1+9 with water to produce 1X tris-borate buffer.

Lysozyme solution. 50 mg/ml in sucrose medium.

Ethanol, 100% and 70% aqueous solution, cooled to 4°C.

T1 RNase solution, 100 U/ml.

RNase A solution, 10 mg/ml.

Proteinase solution, 10 mg/ml.

Agarose gels. Prepare 0.8% gels in 1X tris-borate buffer. A gel 20cm long, 10cm wide and 3-4mm deep requires about 100 ml agarose solution. Use 1.5% agar for end plugs, if required. When the gel has solidified, cover its surface minimally with tris-borate buffer (approximately 40 ml).

PVDC film wrap ("cling-film"), Saran™ or equivalent.

Electrophoresis apparatus, suitable for running agarose gels. BioRad; GE (formerly Pharmacia) or equivalent.

DNA molecular weight marker solution, 1kB ladder (Invitrogen), Pulse marker (Sigma) or equivalent.

Tracking dye for electrophoresis, containing 0.25% bromophenol blue and 15% Ficoll 400.

Ethidium bromide solution, 5 µg/ml in water (note: wear nitrile gloves for handling solution and treated gels).

UV lamp, for visualization of DNA bands.

Method

- i. Aseptically transfer about 100 mg of Bti GR into 2 ml medium/water in a sterile bottle and mix thoroughly.
- ii. Maintaining aseptic conditions, inoculate 100 µl of the suspended Bti cell mixture into 20 ml Luria broth and incubate, with shaking, at 28°C for about 16 hours.
- iii. Sediment the cells in a bench-top centrifuge at maximum speed for 15 minutes and discard the supernatant.
- iv. Add 1 ml TES buffer, vortex to re-suspend the pellet, transfer the suspension to a microcentrifuge tube and sediment the cells for 2 minutes at 5°C. Discard the supernatant.

- v. Add 180 µl sucrose medium and vortex to re-suspend the pellet. Add 20 µl lysozyme solution, mix gently by hand (do not use a vortex mixer) and incubate at 37°C for 60 minutes.
- vi. Add 48 µl NaCl solution, 12 µl EDTA solution and 260 µl SDS-NaCl solution and slowly invert the tube, twice. Incubate the mixture for 10 minutes at 68°C, then stand the tube in ice for 60 minutes. Centrifuge at 4°C for 15 minutes to sediment the cell wall debris and transfer 300 µl of supernatant to another micro-centrifuge tube.
- vii. Add 33 µl sodium acetate solution and 670 µl cold 100% ethanol, vortex to mix and place in the freezer for ≥1 hour. Centrifuge at 5°C for 15 minutes and discard the supernatant.
- viii. Add approximately 200 µl cold 70% ethanol, vortex to mix, then centrifuge at 5°C for 10 minutes and discard the supernatant. Dry the pellet in a vacuum dryer for about 30 minutes. Add 200 µl TES buffer to the dried pellet, vortex to re-suspend it, allow the mixture to stand at room temperature for 15 minutes and then vortex again to mix.
- ix. Add 2 µl T1 RNase solution and 2 µl RNase A solution, mix and incubate the mixture at 37°C for 30 minutes. Add 20 µl proteinase solution, mix and incubate the mixture at 37°C for 1 hour.
- x. Mix 15 µl sample solution with 3 µl tracking dye and transfer the whole to a well in the agarose gel. Include an appropriate amount of DNA molecular weight marker solution, according to the manufacturer's directions, in an adjacent well.
- xi. Run the gel at 50 V for about 15 minutes. Turn off the voltage before removing excess buffer from the surface of the gel and then cover it with PVDC film. Adjust the voltage to give 20 mA current and run the gel overnight (16-17 hours). Reverse the voltage polarity for 30 seconds immediately before switching off and removing the gel.
- xii. Stain the gel in ethidium bromide solution for 20 minutes, with gentle rocking. Destain the gel in 1X tris-borate buffer for 20 minutes, changing the buffer 3 times during this time. Place the gel under a UV lamp and photograph it. Bti strain AM65-52 plasmid should produce 5 fluorescent bands below the chromosomal smear and, depending upon the separation quality of the gel, 1 band may appear above the chromosomal smear. Due to possible conformational changes from super-coiled to relaxed forms of the plasmid during preparation, the actual sizes of the plasmids are best determined by comparison on the same gel with a Bt strain having known plasmid sizes, such as Bti reference standards HD-1 or HD-2.

Note 5 Determination of biopotency

Principle

Biopotency is measured in International Toxic Units (ITU) per mg of product. Biopotency is tested by comparing mosquito larval mortality produced by the product under test with the mortality produced by a spray-dried reference powder* of *Bacillus thuringiensis* subsp. israelensis, using early fourth-instar larvae of *Aedes aegypti* (strain Bora Bora). The toxicity (ITU/mg) of products tested is determined according to the following formula:

$$\text{ITU/mg of product tested} = \frac{\text{reference standard ITU/mg} \times \text{LC}_{50} \text{ (mg/l) standard}}{\text{LC}_{50} \text{ (mg/l) product tested}}$$

Equipment and materials

Top-drive homogenizer or stirrer

Ice bath (container of crushed ice)

Analytical balance (accurate to ± 0.1 mg)

Top-pan balance (accurate to ± 10 mg), preferably with tare facility

Deionised water

* The original reference powder recommended by WHO for this purpose, IPS82 strain 1884 from Pasteur Institute, is no longer available. Until a replacement international reference powder of Bti becomes available, a reference standard of strain AM65-52 may be obtained from Valent Biosciences Corp. for the purposes of testing product compliance with the specification. This reference standard of strain AM65-52 was calibrated against IPS82 strain 1884 and has a biopotency of 7992 ITU/mg.

Wetting agent (e.g. Tween 80)
200 ml beakers, borosilicate glass or plastic
500 ml bottle, wide-necked, screw-capped, clear glass
100 ml bottles, screw-capped clear glass
Micropipette
10 ml pipette
12 ml tubes, plastic with stoppers or caps
200 ml cups, plastic or wax-coated paper

Method

(i) Production of test larvae

L4 larvae are representative of the total sensitivity of the target population and convenient to handle. It is very important to use a homogenous population of early fourth instars, which are obtained within five days of hatching, using standardized rearing methods.

Aedes aegypti eggs are laid in a cup lined with filter paper and one-third filled with deionised water. The paper is dried at room temperature and kept for several months by storing in a sealed plastic bag at room temperature. When larvae are needed, the paper is immersed in de-chlorinated water. To synchronise hatching, add larval feed to the water 24 hours prior to adding the eggs. The bacterial growth will deoxygenate the water and this triggers egg hatching. This usually induces the first instars to hatch within 12 h. These larvae are then transferred to a container (25 x 25 x 10 cm) containing 2 litres of de-chlorinated water, to obtain a population of 500 to 700 larvae per container. Larval feed may be flakes of protein as used for aquarium fish, or powdered cat biscuit, and the containers are held at $25 \pm 2^\circ\text{C}$. It is important that the amount of food is kept low to avoid strong bacterial growth that kills the larvae. Several feeds, at 1- to 2-day intervals and daily observation of the larvae is optimal. If the water becomes turbid, replace all water by filtering out the larvae and transfer to a clean container with clean water and feed. Five to seven days later, an homogeneous population of early fourth instars (5 days old and 4 to 5 mm in length) should be obtained.

(ii) Preparation of reference standard suspensions for calibration of the bioassay

Before preparing the suspension, check that stirring/blending of the wetting agent/water mixture, described in the following paragraph, does not lead to foaming. If it does, dilute (e.g. 1:10) the wetting agent before use.

Accurately weigh about 50 mg (to the nearest 0.1 mg) of the reference standard powder and transfer it to a 200 ml beaker with 100 ml deionised water (it can be transferred directly to the 500 ml bottle if the neck is wide enough to accept the stirrer/blender head). Allow the mixture to stand for 30 min and add a small drop (about 0.2 mg) of wetting agent. Place the beaker in the ice bath and either stir or blend the mixture for 2 min. Check visually for any large particulates remaining and repeat the stirring/blending if there are any. Weigh or tare the 500 ml bottle and transfer the suspension/solution to it, rinsing carefully and thoroughly the beaker and stirrer/blender. Add further deionised water to make the weight of contents to 500 g (500 ml), cap the bottle and shake vigorously to mix the contents. Confirm, by microscopic examination of a small aliquot, that no aggregates of spores and crystals persist. If any are present, the contents must be subjected to further stirring/blending in the ice bath. This primary suspension/solution contains 1 mg/10 ml and must be shaken vigorously immediately before removing aliquots.

Transfer 10 ml aliquots of the primary solution/suspension to clean 12 ml tubes that are stoppered/capped immediately. If transferring a number of aliquots, cap and shake the primary suspension/solution at intervals not exceeding 3 min, because the spores and crystals settle quickly in water. The aliquots can be stored for a month at 4°C and for 2 years in a freezer at -18°C . Each contains 1 mg standard powder.

To prepare a "stock solution", weigh or tare a 100 ml bottle. Transfer one of the 10 ml aliquots into the 100 ml bottle, rinsing carefully at least twice with deionised water, and fill to a total of 100 g. Shake the mixture vigorously (or use the blender) to produce an homogeneous suspension. Frozen aliquots must be homogenised thoroughly before use, because particles agglomerate during freezing. The "stock solution" contains 10 mg/l.

From the “stock solution”, subsequent dilutions are prepared directly in plastic cups filled (by weighing) with 150 ml de-ionized water. To each cup, 25 early L4 larvae of *Aedes aegypti* are added first by means of a Pasteur pipette, prior to addition of bacterial suspensions. The volume of water added with the larvae is removed from the cup (by weighing) and discarded, to avoid changing of the volume of liquid in the cup. Using micropipettes, 600 µl, 450 µl, 300 µl, 150 µl, 120 µl and 75 µl of “stock solution” are added to separate cups and the solutions mixed to produce final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l, respectively, of the reference standard powder. Four replicate cups are used for each concentration and one for the control, which contains only 150 ml de-ionized water.

(iii) Preparation of suspensions of the product to be tested

Weigh the desired amount of granular sample and place the sample in a glass bottle containing 100 ml of 0.2% Tween solution. Place the sample bottle on a wrist-action shaker and shake the sample bottle for a minimum of 20 minutes. Perform the subsequent dilution series in the same manner as described for the reference standard powder, above, except that the replicate determinations must be made on dilutions prepared by weighing separate test portions of the product. That is four replicate primary suspension/solutions must be prepared. Cups and larvae are prepared as described above and comparable dilutions are prepared as for the reference standard.

(iv) Determination of toxicity

No food is added for *Aedes* larvae. All tests should be conducted at $28 \pm 2^\circ\text{C}$, with a 12 h light, 12 h dark cycle. To avoid the adverse effects of evaporation of water in low humidity, the relative humidity should be maintained at $50 \pm 15\%$, if possible.

Each bioassay series should preferably involve 6 concentrations x 4 replicates x 25 larvae for the reference standard and the unknown and 100 larvae for the control. The aim is to identify a range of concentrations that give mortality between 5-95% (because 100 larvae are used). Data giving 0 or 100% mortality are ignored for the calculation of the LC_{50} . To prepare a valid dose-response curve, only concentrations giving between 95 and 5% mortality should be used. Within this range, a minimum of two concentrations must be above the LC_{50} and two below, to ensure the validity of the LC_{50} value (the sensitivity of the insect colony may require a slightly different 6-dilution series to be used).

Mortality is determined at 24 and 48 h by counting the live larvae remaining. If pupation occurs, the pupae should be removed and their numbers excluded from the calculations. If more than 5% of larvae pupate, the test is invalidated because larvae do not ingest 24 hours before pupation and too many larvae may have survived simply because they were too old. Because of the very rapid killing action of Bti, usually there is no difference between the 24 and 48 h mortality. In this case, the 48-h count confirms the 24-h reading and provides a check on the possible influence of factors other than Bti components.

If the control mortality exceeds 5%, the mortalities of treated groups should be corrected according to Abbott's formula [Abbott, W. S., (1925). A method for computing the effectiveness of an insecticide. Journal of Economic Entomology, **18**, 265-267]:

$$\text{percentage (\% control)} = \frac{X - Y}{X \times 100}$$

where: X = % survival in untreated control;
Y = % survival in treated sample.

Tests with a control mortality greater than 10%, or any pupation greater than 5%, should be discarded. Mortality-concentration regression lines may be drawn on gauss-logarithmic paper but this is rather subjective. It is preferable to use a statistical program, such as SAS, which incorporates Log Probit Analysis. With such a statistical program, Abbott's formula is not required because the correction is automatically carried out by the program. The toxicity is determined by estimation and comparison of the LC_{50} s of the tested product and reference standard preparations, using the formula described above. The toxicity is defined by the count at 24 h after initiation of the test.

For increased accuracy, bioassays should be repeated on at least three different days, concurrently with the assay of the reference standard, and the standard deviation of the means calculated. A test series is valid if the relative standard deviation (RSD) is <25%.

Note 6 Beta-exotoxin (a heat-stable nucleotide composed of adenine, glucose and allaric acid) has been shown not to occur in the products of the manufacturer identified in evaluation report 770/2006 and its presence is unlikely to occur spontaneously. However, beta-exotoxin can be generated by some strains of *Bacillus thuringiensis* and, if detectable by the method of Bond *et al.*^{*}, it would be designated as a relevant impurity and a clause would be required to limit its concentration.

Note 8 ***Staphylococcus aureus* Enumeration and Identification**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$
Incubator, controlled to within the range 35-37°C
Soybean Casein Digest Broth with 1% Tween-20, or equivalent
Sterile, pre-poured Baird-Parker agar plates (Difco 0768-01-1, BBL 11023, or equivalent), supplemented with egg yolk tellurite enrichment (Difco 0779-73-1 or equivalent)
Mammalian plasma (rabbit coagulase plasma, lyophilized, BBL 40658 or equivalent)
Sterile bottles, capped, at least 100 ml
Sterile test tubes

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml soybean casein digest broth with 1% Tween-20 (pre-warmed to NMT 45°C). Mix well to disintegrate and disperse the formulation.

In duplicate, transfer 1 ml suspension (0.1 g GR) onto the surface of agar plates. Spread the inoculum evenly across the surface and allow it to sink into the surface of the agar. Cover, invert and incubate the plates for 21-26 hours before examining them.

S. aureus forms black, shiny, convex colonies surrounded by a clear zone on Baird-Parker agar. If negative for *Staphylococcus*, incubate the plates for an additional 24 hours and read again. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. Perform gram stains on a typical suspect colony from each plate. *S. aureus* are gram positive cocci occurring in clusters. If the cells do not conform to this description, *S. aureus* can be considered absent from the sample. However, if the cells conform to the description, perform the following test.
- ii. Coagulase test. Using a sterile inoculating loop, transfer a portion of a typical suspect colony to 0.5 ml mammalian plasma in a sterile test tube, and swirl to mix. Conduct the test in parallel with positive and negative controls. Place the tubes in the incubator, examine them after 3 hours and then at suitable intervals for a total of 24 hours. The positive and negative controls should show coagulation and no coagulation, respectively. If the test of the suspect colony shows no visible coagulation, it can be concluded that coagulase positive *S. aureus* is absent.

Note 8 ***Salmonella* species detection**

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$
Incubator, controlled to within the range 35-37°C
Soybean Casein Digest Broth w/ 1% Tween 20, or equivalent

^{*} Bond R.P.M., *et al.* The thermostable exotoxin of *Bacillus thuringiensis*. In: Burges H. D. and Hussey N. W., eds. Microbial control of insects and mites. Academic Press, London, 1971.

Selenite-cystine broth, 10 ml capped test tubes (Difco 0687, BBL 11606, or equivalent)
Sterile, pre-poured brilliant green agar plates, (Difco 0014, BBL 11073, or equivalent)
Sterile, pre-poured xylose-lysine-desoxycholate (XLD) agar plates, (Difco 0788, BBL 11838, or equivalent)
Sterile, pre-poured bismuth sulfite agar plates, (Difco 0073, BBL 11031, or equivalent)
Sterile, pre-prepared butt-slant tube containing approximately 10 ml triple sugar iron agar (Difco 0265, BBL 11749, or equivalent)
Brilliant green solution, USP (1:1000 aqueous solution prepared and stored at 2-8°C)
Iodine-iodide solution, USP (Dissolve 5 g potassium iodide and 6 g iodine in 20 ml USP purified water; store at 2-8°C)
Fluid tetrathionate broth (Difco 0104, BBL 11705, or commercially-prepared equivalent), 10 ml in capped test tubes. To each 10 ml tetrathionate broth tube, add 0.1 ml of the prepared brilliant green solution; mix, then add 0.2 ml prepared iodine-iodide solution. Mix.

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml soybean casein digest broth with 1% Tween 20 (pre-warmed to NMT 45°C). Mix well to disintegrate and disperse the formulation and incubate for 24 hours.

Transfer 1 ml portions (0.1 g GR) of the incubated culture into two separate tubes containing, respectively, 10 ml selenite cystine broth and 10 ml fluid tetrathionate broth containing iodide-iodine solution and brilliant green solution. Mix and incubate the inoculated tubes for 18-24 hours.

Using an inoculating loop, streak portions of the incubated selenite cystine and tetrathionate tubes onto separate plates of brilliant green agar, XLD agar, and bismuth sulfite agar. Cover, invert and incubate the plates for 18-24 hours, or for at least 48 hours in the case of bismuth sulfite agar, before examining them.

Salmonella colonies exhibit the following characteristics.

Brilliant green agar – small transparent colourless, or pink/white opaque, colonies surrounded by a pink to red zone.

XLD agar – red colonies, with or without black centres.

Bismuth sulfite agar – metallic black or dark green colonies.

If none of the colonies match these descriptions, the sample meets the requirement for absence of Salmonella species. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. If colonies matching any of the above descriptions are found, perform a gram stain on material taken from them. Salmonella species are gram negative rods. If the cells match this description, proceed with confirmatory test (ii), below.
- ii. With an inoculating needle or loop, transfer material from the suspect colonies to a butt-slant tube containing triple sugar iron (TSI) agar. First stab the needle/loop beneath the surface, then streak the slant. Incubate the tube(s) for 12-24 hours. Salmonella species typically ferment glucose with the production of acid and some species also produce gas and H₂S (Table 1).

The tube, if positive for Salmonella, will have an alkaline (red) slant and an acid (yellow) butt with or without blackening of the butt from H₂S production. If the presence of Salmonella is indicated, proceed further to identify the organism by employing the Vitek/Vitek2 automated microbial identification system, other approved identification system, or by performing appropriate biochemical and cultural reactions.

Table 1. Reactions observed in TSI agar

Reaction	Explanation
Acid butt (yellow), alkaline slant (red)	Glucose fermented
Acid throughout medium, butt and slant yellow	Lactose or sucrose, or both, fermented
Gas bubble in butt, medium sometimes split	Aerogenic culture
Blackening in the butt	Hydrogen sulfide produced
Alkaline slant and butt (medium entirely red)	None of the three sugars fermented

Note 9 *Pseudomonas aeruginosa* Enumeration and Identification

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$.

Incubator, controlled to within the range 35-37°C.

Filter papers.

Sterile phosphate buffer, pH 7.2 (USP or equivalent) with 1% Tween-80, or equivalent

Sterile, pre-poured cetrimide agar plates, (BBL 11554-pseudoseal agar, or equivalent).

N,N-dimethyl-p-phenylenediamine dihydrochloride.

Oxidase reagent (DrySlide® BBL 231746, or equivalent).

Procedure

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml sterile USP phosphate buffer with 1% Tween-80 (pre-warmed to NMT 45°C). Mix well to disintegrate and disperse the formulation.

Transfer, in duplicate, 1 ml portions (0.1 g GR) of the suspension onto the surface of cetrimide agar plates. Spread the inoculum evenly across the surface of the plates, cover and allow the inoculum to sink into the surface. Invert and incubate the plates for 48-72 hours before examining them.

P. aeruginosa forms characteristic bluish or greenish colonies. Apparent positives should be subjected to confirmatory tests.

Confirmatory tests

- i. Perform a gram stain. *P. aeruginosa* cells are slender, gram negative rods.
- ii. Perform an oxidase test.

Either Using a platinum wire loop or sterile wooden stick, transfer a portion of the suspect colony to a DrySlide® oxidase reaction area. Spread the inoculum on the reaction area to a 3-4 mm size. Examine the reaction area within 20 seconds. Positive reaction: organisms produce a purple to dark colour within 20 sec. Negative reaction: organisms produce no colour change, or a change to light grey, within 20 sec.

Or Use filter paper impregnated with either *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride or wetted with a drop of oxidase reagent. Concurrently perform the test on a *P. aeruginosa* reference culture, as a positive control. If a purple colour does not develop within 30 seconds, the result is negative.

- iii. If necessary, additional confirmation may be sought using biochemical or cultural tests appropriate for the identification of oxidase-positive, gram-negative, non-fermenting rods.

Note 10 *Escherichia coli* enumeration (pour plate method)

Equipment and materials

Balance, with tare, capable of weighing 10 g to within $\pm 1\%$.

Incubator, controlled to within the range 35-37°C.

Long-wavelength UV light source.

Soybean Casein Digest Broth with 1% Tween-20, or equivalent

Violet red bile agar with 4-methylumbelliferyl- β -D-glucuronide (VRB agar with MUG) (Difco 229100 or equivalent).

Sterile Petri dishes.

Method

Observe aseptic precautions and handling throughout.

Open the bag containing the formulation and transfer 10 g into 90 ml soybean casein digest broth with 1% Tween-20 (pre-warmed to NMT 45°C). Mix well to disintegrate and disperse the formulation. If necessary, dilute further with sterile phosphate buffer, with thorough mixing, so that 1 ml will yield not more than 300 colonies. In duplicate, transfer 1 ml suspension (0.1 g GR if the suspension has not been further diluted) into each of two sterile Petri dishes. Add to each dish approximately 15-20 ml of VRB agar with MUG that has been cooled to about 45°C.

Cover the Petri dishes, mix the suspension with the agar by rotating the dishes in one direction and then in the opposite direction. Allow the contents to solidify at room temperature, invert the plates and incubate at 35-37°C for 20-24 hours.

Examine the plates for growth in the dark using the UV light source, for fluorescent colonies. Typical strains of *E. coli* (red colonies surrounded by a bile precipitate) exhibit a bluish fluorescent halo (MUG-positive). Apparent positives should be subjected to confirmatory tests. If confirmed as *E. coli*, count the number of MUG-positive colonies and calculate the average count for the two plates. Do not count colonies of non-*E. coli* coliforms, which may also produce red colonies with zones of precipitated bile but are MUG-negative.

Confirmatory tests

- i. Presumptive colonies of *E.coli* should be confirmed using either a Vitek automated microbial identification system or by performing other appropriate biochemical and cultural tests to confirm the presence of *E. coli*.

Note 11 Measurement of dustiness must be carried out on the sample "as received" and, where practicable, the sample should be taken from a newly opened container, because changes in the water content of samples may influence dustiness significantly. The optical method, MT 171.2, usually shows good correlation with the gravimetric method, MT 171.1, and can, therefore, be used as an alternative where the equipment is available. Where the correlation is in doubt, it must be checked with the formulation to be tested. In case of dispute the gravimetric method shall be used.

Note 12 Tests for bacterial contaminants (clauses 4.1-4.4) are not specified after storage of the product for 14 days at 54°C, because this regime is unlikely to reveal the extent of potential proliferation that might occur under normal storage conditions.

Note 13 Samples representing "before" and "after" the storage stability test should be tested concurrently after the test, in order to minimize the variation occurring in assays of the biopotency. Material for the "before" test sample should be stored in a sealed container at 2-8°C, for the duration of the test, prior to bioassay. If the container is stored for this purpose in a refrigerator or freezer, it must be equilibrated to room temperature and dried externally before opening, to avoid contaminating the granules with atmospheric moisture which could affect the results of tests such as biopotency and dustiness.

PART TWO
EVALUATION REPORTS

***Bacillus thuringiensis subsp. israelensis* strain AM65-52**

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WHO SPECIFICATIONS AND EVALUATIONS FOR PUBLIC HEALTH PESTICIDES

***Bacillus thuringiensis subsp. israelensis* strain AM65-52**

FAO/WHO EVALUATION REPORT 770/2012

Recommendation

The Meeting recommended that the new specification for *Bacillus thuringiensis* subspecies *israelensis* (Bti) strain AM65-52 granules, proposed by Valent BioSciences and as amended, should be adopted by WHO.

Appraisal

The data were submitted in 2010 and were broadly in accordance with the requirements of the 2010 revision of the FAO/WHO Manual and supported the draft specifications for new WHO specifications for a granular formulation (GR) of *Bacillus thuringiensis* subspecies *israelensis* strain AM65-52 (*Bti* AM65-52). The product is a bacterial larvicide intended to be applied directly to the habitats of the mosquito larvae in open bodies of water, but the product is not intended for use against container breeding mosquitoes or for addition to potable water (see below). The product has been tested and recommended by WHOPES in 2012 (WHO 2012).

Identity, description clause

As explained in the 2006 FAO/WHO evaluation report, *Bti* AM65-52 is a bacterial larvicide consisting of a mixture of crystalline inclusions (insecticidal proteins), cells and spores of the *Bti* AM65-52 strain. *Bti* AM65-52 is produced in a closed system and the active ingredient complex is not isolated. Therefore, a TC or TK in proper sense cannot be referenced to in the description clause. The standard description clause refers to application of granules by machine, whereas the *Bti* AM65-52 GR formulation can be applied by machine as well as manually.

Identity tests, content of active ingredient, water as relevant impurity and bacterial contaminants

The identity tests are based on a series of increasingly complex tests starting with a rather simple microscopic examination to the more demanding electrophoresis tests and are the same as those for the WG formulation. The content of active ingredient or rather biopotency of *Bti* AM65-52 GR is 200 ITU/mg and determined using the 4th instar larvae of *Aedes aegypti* toxicity test *in vitro*. As in the WG formulation, water was proposed and accepted as relevant impurity as being present in higher amounts could destabilize *Bti* AM65-52.

The Meeting concluded that that human pathogens in the *Bti* AM65-52 end use product are considered as relevant impurities which may increase or extend the hazard of the product and therefore have to be limited to a certain level.

The absence of three different pathogens are to be verified (no detection of *Staphylococcus aureus*, *Salmonella* species and *Pseudomonas aeruginosa*) when samples of the product are examined by classical microbiological techniques on selective media and colony counting. A maximum of 100 colony-forming units is tolerated for *Escherichia coli*.

Physical properties and storage stability

The clauses, limits and test methods proposed for physical properties of the GR were in accordance with the requirements of the Manual (FAO/WHO, 2010) with a minor deviation: the results of the particle size distribution using MT 170 were expressed in mesh instead of μm , which was then corrected. The formulation is nearly dust-free, but the limit for attrition resistance of 97% was considered rather low and questioned by the Meeting. The manufacturer explained that the granules are rather soft to ensure a rapid release of Bti, Strain AM65-52 from the granules and this was accepted by the Meeting. The biopotency after accelerated storage stability shows a more pronounced decrease in the GR (a minimum of 70 % remaining after 2 weeks at 54 °C) as compared to 84 % in the WG formulation. The manufacturer explained that the GR formulation has a different composition than the WG and apparently the decrease in biopotency is related to some catalytic activities of formulants on insecticidal proteins. The meeting accepted the explanation.

As with the WG formulation, the bacterial contaminants are not expected to increase in storage, therefore a clause to limit the occurrence of *E. coli* after storage was considered unnecessary.

Physical properties of the WG

The Meeting agreed also to update in the WG specification the CIPAC methods for some physical properties (wet sieve test - MT 185 instead of MT 167 and suspensibility - MT 184 instead of MT 168) to be in line with the WG specification guideline of the November 2010 – second revision of the first edition of the FAO/WHO Manual and the CIPAC methods actually recommended.

ANNEX 1. REFERENCES

Study number	Author(s)	Year	Study title. Study identification number. Report identification number. GLP [if GLP]. Company conducting the study
	FAO/WHO	2010	Manual on development and use of FAO and WHO specifications for pesticides. Second revision of the 1 st edition. FAO, Rome and WHO, Geneva, November 2010 (internet publications).
	WHO	2005	Report of the Nine WHOPES Working Group Meeting, WHO/HQ, Geneva, 5-9 December 2005. WHO, Geneva, document WHO/CDS/NTD/WHOPES/2006.2
	WHO	2012	Report of the Fifteenth WHOPES Working Group Meeting, WHO/HQ, Geneva, 18-22 June 2012. WHO, Geneva, ISBN 978 92 4 150408 9 and WHO/HTM/NTD/WHOPES/2012.5

WHO SPECIFICATIONS AND EVALUATIONS FOR PUBLIC HEALTH PESTICIDES

***Bacillus thuringiensis subsp. israelensis* strain AM65-52**

FAO/WHO EVALUATION REPORT 770/2006

Recommendation

The Meeting recommended that:

- (i) the specification for *Bacillus thuringiensis subspecies israelensis* (Bti) strain AM65-52 water dispersible granules (WG), proposed by Valent BioSciences Corp. and as amended, should be adopted by WHO;
- (ii) a new internationally validated reference material of Bti should be developed to support the WHO specification for Bti.

Appraisal

The data for *Bacillus thuringiensis subsp. israelensis* strain AM65-52 (Bti AM65-52) were evaluated in support of a new WHO specification for the WG formulation.

The draft specification and supporting data were provided by Valent Biosciences Corp., USA, over the period 2003-2006, during which various aspects of the specification and the supporting test methods were elaborated.

Bti AM65-52 is a bacterial larvicide for mosquito control, the strain originally having been isolated from a natural population of *Bacillus thuringiensis*.

Bti AM65-52 is not under patent.

Many of the physical and chemical parameters used to characterize synthetic and natural chemicals are inappropriate for micro-organisms, such as Bti, but the principles underlying FAO and WHO specifications for pesticides remain applicable to products based on living organisms.

Bti AM65-52 WG is formulated from the active ingredient in a closed system, with no isolation of a technical product, and thus a specification for the corresponding technical grade active ingredient was neither practical nor proposed. Supporting data on hazards, produced using specially prepared technical grade Bti A65-52, were provided by the manufacturer and the Meeting agreed that, in this case, a specification could be developed for the WG in the absence of a specification for technical grade active ingredient. The Meeting noted that a specification could be developed subsequently for technical grade Bti AM65-52, if required.

A summary of commercially confidential information on the manufacturing process, data from 5-batch analyses and the manufacturing specification were provided to WHO. Complete chemical characterization of Bt batches is neither possible nor appropriate and therefore mass balances could not be estimated. Only general information was provided on the complex natural products used in culturing the Bti but the manufacturer subjected the constituents to quality checks (details not provided) and the Meeting considered that impurities introduced from this source were unlikely to be a cause for concern. Although a detailed comparison could not

be made for administrative reasons, US EPA confirmed that the data provided to WHO were in line with those submitted for registration in the USA.

WHO/PCS advised that the hazard data on Bti AM65-52 were in accord with the many other published data on Bti and Bti products and hence gave no cause for concern. The Meeting noted the absence of data on the mutagenicity of Bti AM65-52. The manufacturer stated that the absence of exotoxin or other Bti components known to bind to DNA had led regulatory authorities (EU, directive 2001/36/EC; PMRA Canada, 2001 Guidelines for the Registration of Microbial Pest Control Agents and Products) to decide that genotoxicity testing is not required for Bti AM65-52. The US EPA and IPCS had also concluded that, with the exception of eye and skin dermal irritation, none of the Bt hazard studies demonstrated any clear risk to human health, even when it is present in drinking-water or food. In a standard test for the assessment of dermal sensitization by chemicals, a positive response was recorded. The Meeting agreed that the hazard data package provided was sufficient for the evaluation.

Description clause and header note

As indicated above, the description clause cannot cross-reference a corresponding technical grade active ingredient, because this is not isolated. For this reason, the revised (2006) standard header note for FAO/WHO formulation specifications (other than slow-release formulations), which permits any formulator using TC/TK from the evaluated source to utilize the specification, cannot be applied. The previous header note, restricting application to the evaluated formulation was therefore retained.

The standard description clause was also amended to reflect the fact that the WG be applied either directly to a body of water or dispersed in water for spray application.

Identification and quantification

Bti AM65-52 spores in the WG are not inactivated and, as proliferation of Bti cells may have a secondary role in the biological activity, the Meeting agreed that the active ingredient should be defined as the mixture of crystalline inclusions (insecticidal proteins), cells and spores of the Bti AM65-52 strain.

Identification of the active ingredient is problematic. Microscopic examination of the cells can establish that they belong to the broad group of gram-positive, motile, rod-shaped bacteria. Similarly, the presence of spores and protein crystals may suggest that the bacteria are Bti. Microscopic examination therefore provides a simple and rapid initial screen for identification. Bti may be identified by the flagella antigen (H-14) but, unfortunately, well-characterized specific antibodies (or antisera) are not available commercially and the manufacturer was unable to provide them for routine testing. Production of such antibodies is beyond the capabilities of most testing laboratories but, if available, they could be used to confirm the presence of Bti. Bti strain AM65-52 is best identified by SDS-PAGE of the crystal proteins produced by it and/or agarose GE of the plasmid DNA encoding the proteins. Both test methods are well-established and widely used in many similar applications.

Bti cells and spores are necessary components of the active ingredient but the number of Bti cells and/or spores is not necessarily a good indicator of the larvicidal activity of any Bt product. The Meeting therefore agreed that it is neither necessary nor appropriate to specify minimum contents of cells or spores for the WG. IPCS concluded (IPCS 1999) that Bti spores and cells present no significant hazards to users or the environment and therefore the Meeting agreed that it is not necessary to

specify maximum content values for the WG. The content of insecticidal crystal proteins could be determined by HPLC. However, bioassay, using “standardized” mosquito larvae, is generally accepted as the most reliable means for establishing the content of active ingredient. The bioassay is a standard WHO test method but, unfortunately, the reference Bti material recommended by WHO for calibration (IPS82 strain 1884) is no longer available. Valent Biosciences Corp. proposed that the company should make available a reference standard of strain AM65-52 for the purposes of testing product compliance with the proposed WHO specification. The reference standard of strain AM65-52 (lot #82-691w5) had been calibrated by the company against IPS82 strain 1884 and has a biopotency of 7992 ITU/mg. The Meeting acknowledged that the company’s reference standard had not been independently assessed and calibrated but it was considered that, until a replacement international reference Bti powder becomes available, the Valent BioSciences reference standard should be used for testing compliance with the specification.

Relevant impurities

The Meeting agreed that control of water content in the WG is essential for maintenance of product quality, and accepted the proposed limit of 50 g/kg. The test method is a standard CIPAC method.

Beta-exotoxin was initially proposed as a relevant impurity but, being produced only by certain other strains of Bt, it should not occur in pure cultures of Bti. The manufacturer stated that the Bti AM65-52 WG under consideration is routinely tested for the presence of beta-exotoxin but it has never been detected. The Meeting therefore agreed that it should not be designated as a relevant impurity but that a footnote should be appended to the specification cautioning that, if beta-exotoxin is detectable in superficially similar products from other manufacturers, it should be considered a relevant impurity in such products. A standard method is available for assay of beta-exotoxin.

Bacterial contaminants

The Meeting sought the advice of WHO/PCS on the subject of specification clauses to limit bacterial contaminants (i.e. bacteria other than Bti AM65-52), to resolve several issues. Unlike most formulated synthetic pesticides, Bti AM65-52 WG forms a medium in which, in the presence of water, various bacteria may proliferate. Rates of proliferation would be highly dependent upon local conditions, and therefore impossible to predict in any particular application, but the WG is intended for use in potable water supplies. Although it could be argued that users themselves are also likely to be sources of bacterial contamination, WHO/PCS advised that important pathogenic bacteria must be controlled in any product which may be added to potable water.

In terms of the species to be controlled, the manufacturer initially proposed clauses for *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, total coliforms, *Salmonella* species and total enterococci. WHO/PCS considered that clauses for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella* species are essential. WHO/PCS also considered that a clause for *Escherichia coli* is required, given the high pathogenicity of certain strains. Because *E. coli*, total coliforms and total enterococci all provide adequate markers of faecal contamination, the manufacturer and the Meeting agreed that control of *E. coli*, alone, should be

sufficient for this purpose. WHO/PCS questioned the manufacturer's subsequent withdrawal of the proposed clause for *Clostridium perfringens* but it was explained that this species is unable to proliferate in the medium used to grow Bti AM65-52. The Meeting therefore agreed that clauses were required to control *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* species and *Escherichia coli*.

Setting appropriate limits for these four bacterial contaminants was particularly problematic. WHO limits for the presence and numbers of bacteria in drinking water are only available for *E coli* (as an indicator of faecal contamination). Of course, Bti AM65-52 WG is not intended for direct consumption, so it is difficult to make a comparison with the standard set for drinking water. Sampling, the numbers of replicate tests required and the calculation of bacterial concentrations were also issues where comparisons between drinking water and the use of Bti AM65-52 WG proved problematic.

Ultimately, and on a practical and pragmatic basis, WHO/PCS, the manufacturer and the Meeting agreed that limits for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella* species should be in the form of "...not detected when tested by the method described in a footnote". For *E coli*, it was agreed that the limit should be "...<100 CFU/g when tested by the method described in a footnote". In likely use and exposure scenarios, this limit is at least as stringent as the WHO guideline for drinking-water quality.

The test methods for bacterial contaminants are essentially standard bacterial tests.

Physical properties

The clauses, limits and test methods proposed for physical properties of the WG were in accordance with the requirements of the Manual (FAO/WHO, 2006).

Storage stability

The manufacturer provided data on the stability of Bti AM65-52 WG stored at -18, +20 and +25°C for 6, 12, 18 and 24 months but the 2004 Meeting agreed that an accelerated storage stability test was required. The Meeting acknowledged that simple extrapolation from these data, using the Arrhenius equation, would be wholly inappropriate for such a complex mixture as Bti AM65-52 WG, because of the myriad of (mostly) unknown reactions involved in the degradation. The manufacturer therefore conducted trials on samples of standard, within-specification WG at 54°C for up to 4 weeks, encompassing the standard 2-week period of CIPAC MT 46.3. After 2 and 4 weeks at 54°C, potency of the WG declined by 15-16% and 21%, respectively, although it remained above the specified minimum of 2700 ITU/mg. After 1 and 2 weeks at 54°C, the physical properties were not significantly altered. The manufacturer proposed a limit of 84% for retention of potency after 2 weeks at 54°C, with no change in physical properties, and this was accepted by the Meeting.

On the basis of advice received from WHO/PCS and by WHOPES, the Meeting agreed that bacteria are more likely to decline, than to proliferate, under the conditions of the test and therefore testing for bacterial contaminants after the storage stability test could be misleading. The manufacturer's data indicated no increase in bacterial contaminants during prolonged storage of sealed bags under normal conditions.

**SUPPORTING INFORMATION
FOR
EVALUATION REPORT 770/2006**

Overview of uses

Initially, conventional Bt products were targeted primarily against lepidopteran pests of agricultural and forestry crops but Bt strains active against coleopteran pests are now available. Strains of Bti active against dipteran vectors of parasitic and viral diseases are used in public health programmes. Bti AM65-52 is used in public health applications, to control the larvae of mosquitoes and black flies, the adults of which are disease vectors. The activity of Bti AM65-52 against larvae of *Aedes*, *Culex*, *Anopheles* and *Uranotaenia* mosquitoes was demonstrated many years ago (Golberg & Margalit 1977).

Generally, Bt formulations may be applied foliage, soil, aquatic environments, and food- or water-storage facilities. Formulated as a WG, Bti AM65-52 is intended for mosquito control in potable or non-potable water and may be dispersed in water before or after application.

Most Bt products, including the WG of Bti AM65-52, contain insecticidal crystal proteins and viable spores but in certain Bti products the spores are inactivated.

Resistance to Bt products has been observed in agriculture, indicating the need to avoid indiscriminate heavy use and to adopt good pest management practices.

Identity

Scientific name

Bacillus thuringiensis subspecies *israelensis* strain AM65-52.

Shorthand terms

Bt: all subspecies of *Bacillus thuringiensis*;

Bti: all strains of *Bacillus thuringiensis* subspecies *israelensis* (flagella serotype H-14);

Bti AM65-52: the strain to which CIPAC code 770 applies and the subject of the current evaluation.

CIPAC code number

770

Identity tests

(i) Microscopic examination: gram-positive rods; presence of spores and parasporal crystalline inclusions.

(ii) SDS-PAGE analysis of molecular weight profile of the endotoxin protein crystals.

(iii) Agarose-GE analysis of the plasmid profile.

Definition of active ingredient

A mixture of free endotoxin protein crystals produced by Bti AM65-52 and spores and cells containing them.

Measurement of active ingredient activity

Bioassay with 4th instar larvae of *Aedes aegypti* (strain Bora Bora), results expressed as international toxic units (ITU)/mg product, relative to a reference Bti material. Note: the only reference standard currently available is Valent BioSciences Corp. strain AM65-52, lot #82-691w5, which has a biopotency of 7992 ITU/mg.

Overview of Bt biology (IPCS 1999)

Bt is a facultative anaerobic, motile, spore-forming, gram-positive bacterium found in soils, water, on leaf surfaces in many parts of the world. The coleopteran- and lepidopteran-active Bt subspecies are primarily associated with soil and the phylloplane (leaf surfaces), whereas dipteran-active Bt subspecies are commonly found in aquatic environments. Bt spores are persistent in the environment and vegetative growth occurs when conditions are favourable and nutrients are available. After the application of a Bt subspecies to an ecosystem, the vegetative cells and spores may persist, at gradually decreasing concentrations, for weeks, months or years as a component of the natural microflora. However, insecticidal crystal proteins (ICPs) associated with the spores are rendered biologically inactive within hours or days.

Bt is genetically similar to *Bacillus cereus* (Bc) but distinguished by the formation of characteristic crystalline inclusions, adjacent to the endospore, during sporulation stages III to IV. The parasporal crystalline inclusions consist of one or more insecticidal crystal proteins (ICP) which are toxic to certain invertebrates, especially species of insect larvae belonging to the insect orders Coleoptera, Diptera and Lepidoptera. The crystals have various shapes (bipyramidal, cuboid, flat rhomboid, spherical, or composites of two crystal types), depending on their ICP composition. The crystals of Bti strain AM65-52 occur as irregularly round inclusions. Crystal morphology, ICP composition and specificity of activity against different insect species are broadly correlated.

The basic phenotypic taxon of Bt is the subspecies, of which very many have been described and which are differentiated as serotypes by their flagellar (H) antigens. Most subspecies used for pest control have been isolated from dying insects. The genes encoding ICPs are mostly located on plasmids and are designated by the term *cry* (crystal). Each ICP is the product of a single *cry* gene. *Cry* gene types may be specific to Lepidoptera (*cryI*), Diptera and Lepidoptera (*cryII*), Coleoptera (*cryIII*), Diptera (*cryIV*), or Coleoptera and Lepidoptera (*cryV*), designated according to the classification of Höfte & Whiteley¹. The ICPs of Bti and some other Bt subspecies also incorporate a non-specific cytolytic protein, the gene(s) for which have the designation *cyt* (cytolytic). The crystals of Bti strain AM65-52 contain 4 major proteins, designated Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa according to the classification of Crickmore *et al.* (1998)².

Most plasmids with ICP genes are readily transferred by conjugation between Bt strains and may be transferred to related species of bacteria. Genetic engineering of plasmids has led to the development of strains with novel insecticidal activity. Plasmid genes have also been genetically engineered into plants, for control of plant pests by expression of ICPs within leaf cells, but these are not considered in this evaluation.

¹ Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**: 242-255.

² Crickmore *et al.* (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**: 807-813. Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa correspond to the *CryIVA*, *CryIVB*, *CryIVD* and *CytA*, respectively, of Höfte & Whiteley 1989.

For insect control, sporulated Bt containing ICP or spore-ICP complexes must be ingested by a susceptible insect larva. The ICP (which is a protoxin) is solubilized in the midgut of the larva and converted to the biologically active toxin by proteolytic enzymes. The C-terminal and middle domains of the activated toxin bind to specific epithelial cell membrane receptors in the larval gut, while the N-terminal domain initiates ion channel and pore formation in the membrane, which is followed by consequent lysis of the cell. The mixing of haemolymph and gut contents creates favourable conditions for Bt spore germination and vegetative proliferation, which may result in septicaemia and contribute to the cause of death. Receptor binding by the ICP is the major determinant of host specificity by the different Bt ICPs.

During vegetative growth, various Bt strains are capable of producing an assortment of antibiotics, enzymes, secondary metabolites and toxins, including Bc toxins, that may have detrimental effects on both target organisms and non-target organisms. Of particular note is beta-exotoxin, which is associated with certain Bt subspecies (subspecies *darmstadiensis*, Btd; subspecies *galleriae*, Btg; subspecies *tenebrionis*, Btte; and subspecies *thuringiensis*, Btt). Beta-exotoxin is a heat-stable nucleotide (MW 701) composed of adenine, glucose and allaric acid, which inhibits RNA polymerase by acting competitively with ATP. RNA synthesis is a vital process in all life and therefore beta-exotoxin is toxic towards almost all forms of life, including humans and target insects. Beta-exotoxin is not produced by pure cultures of Bti.

Composition and properties

Table 1. Composition and properties of Bti AM65-52 formulated as WG

Manufacturing process, data on components, impurities and contaminants	Confidential information supplied and held on file by WHO.
Declared minimum Bti AM65-52 content	2700 ITU/mg
Relevant impurities ≥ 1 g/kg and maximum limits for them	Water, 50 g/kg
Relevant impurities < 1 g/kg and maximum limits for them	none
Relevant microbial contaminants and maximum limits for them	<i>Staphylococcus aureus</i> , not detected <i>Salmonella</i> species, not detected <i>Pseudomonas aeruginosa</i> , not detected <i>Escherichia coli</i> , not more than 100 colony-forming units/g
Stabilizers or other additives and maximum limits for them	none

Hazard summary

The standard tests of sub-acute to chronic toxicity, used for synthetic chemicals, are not entirely appropriate for microbial pesticides, which are regulated primarily on the basis of pathogenicity studies (for example, PMRA Canada: DIR 2001-2. EU: Annex 6b of Directive 91/414/EEC). The toxicology of microbial pesticides is considered but pathogenicity potential, infectivity and the pattern of clearance are at least as important.

Many data on the hazards of Bt and Bti are available from the open literature.

Bacillus thuringiensis ssp. *israelensis* (Bti) has been evaluated by the IPCS (IPCS 1999) and the US EPA (USEPA, 1998). *Bacillus thuringiensis* ssp. *israelensis* strain AM65-52 was reviewed for re-registration by the US EPA in 2006. This strain is also under review by the European Commission, with completion scheduled for late 2008.

The IPCS concluded as follows (IPCS 1999). "For aeons, humans have been exposed to Bt in their natural habitats, particularly from soil, water and the phylloplane. However in the recorded scientific literature, only few adverse effects to these environmental Bt levels have been documented. Owing to their specific mode of action, Bt products are unlikely to pose any hazard to humans or other vertebrates or to the great majority of non-target invertebrates, provided that they are free from non-Bt micro-organisms and from biologically active products other than the ICPs. Bt products may be safely used for the control of insect pests of agricultural and horticultural crops as well as forests. Bt is also safe for use in aquatic environments including drinking-water reservoirs for the control of mosquito, black fly and nuisance insect larvae. However, it should be noted that vegetative Bt have the potential for the production of Bc-like toxins, the significance of which as a cause of human disease is not known...[, although]...commercial Bt products do not contain metabolites that are considered hazardous to humans and the environment."

WHO and the EU (under Council Directive 67/548/EEC) have given no hazard classification to Bti. The US EPA has exempted Bti from a requirement for tolerances, it has concluded that Bt products pose no threat to groundwater and has also not issued restrictions on the use of Bt around bodies of water.

Formulations

The main formulation type available is WG. Bti AM65-52 is not co-formulated with other pesticides. The WG is registered and sold in Algeria, Argentina, Australia, Brazil, Canada, Mexico, New Zealand, Singapore, Turkey and the USA.

Methods of analysis and testing

The method for determination of the active ingredient content is bioassay of activity towards mosquito larvae, which is a standard WHO method. The Bti is determined as biopotency, comparing mosquito larval mortality produced by the product under test with the mortality produced by a corresponding reference standard. Biopotency is measured in International Toxic Units (ITU) per mg of product. The biopotency of Bti is compared against a lyophilized reference powder of this bacterial species, using early fourth-instar larvae of *Aedes aegypti* (strain Bora Bora). The toxicity of the first reference standard was originally arbitrarily assigned a toxicity of 15,000 ITU/mg powder against this insect strain. The bioassay provides supporting information in the identification of Bti, due to the specificity of Bti to Diptera.

Identification of the active ingredient depends upon a series of tests, in addition to the quantitative test mentioned above. Microscopic examination is a simple and rapid initial screen for identification, used to establish that the bacterial cells are gram-positive, motile rods, with the presence of spores and protein crystals providing evidence for Bti. In principle, Bti (but not the strain) may also be identified by the flagella antigen (H-14) but suitable antisera are not commercially available at

present. Bti strain AM65-52 is identifiable by SDS-PAGE of the crystal proteins produced by it and/or agarose GE of the plasmid DNA encoding the proteins.

Test methods for the bacterial contaminants, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* species and *Escherichia coli*, involve standard bacteriological techniques.

Test methods for determination of physico-chemical properties of the WG were CIPAC, as indicated in the specification.

Physical properties

The physical properties, the methods for testing them and the limits proposed for the WG formulations, comply with the requirements of the FAO/WHO manual (FAO/WHO 2006).

Containers and packaging

Packaging must be impervious to moisture and light.

Expression and measurement of the active ingredient

The active ingredient, Bti strain A65-52, is defined as a mixture of free endotoxin protein crystals and the Bti cells containing, and spores associated with, endotoxin crystals. The content of active ingredient (biopotency) is measured and expressed as International Toxic Units (ITU) per mg of product. Biopotency is measured by comparing mosquito larval mortality produced by the product under test with the mortality produced by a lyophilized reference powder, using early 4th-instar larvae of *Aedes aegypti* (strain Bora Bora). The original reference strain (IPS82, Bti strain 1884, which is no longer available) had an arbitrarily assigned toxicity of 15,000 ITU/mg powder against this insect strain. Until a new international reference material becomes available to support the WHO specification, Valent Biosciences Corp. has undertaken to provide a reference standard of strain AM65-52 (lot #82-691-W5), which has a biopotency of 7992 ITU/mg.

ANNEX 1

HAZARD SUMMARY PROVIDED BY THE PROPOSER

Note: Valent BioSciences Corp. provided written confirmation that the toxicological and ecotoxicological data included in the following summary were derived from technical Bti AM65-52 having impurity and microbial contaminant profiles corresponding to those of the WG formulation, referred to in Table 2 above, although the technical grade active ingredient is not normally isolated as such.

Table A. Toxicology profile of Bti strain AM65-52 technical material, based on acute toxicity, irritation and sensitization.

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Rat, Sprague-Dawley (5 m, 5 f)	Acute oral	Fasted rats, gavage. US EPA FIFRA, subdivision F, §81-1 ITU/mg not recorded*.	LD ₅₀ >5000 mg/kg bw. No mortality, no significant clinical or body weight effects, no abnormal tissues at necropsy.	6314-95-0090-TX-001
Rabbit, albino (5 m, 5 f)	Acute dermal	US EPA FIFRA, subdivision F, §81-2 ITU/mg not recorded*.	LD ₅₀ >5000 mg/kg bw. No mortality. Soft faeces and ano-genital staining in several rabbits, mainly on day 0-2, body weight not adversely affected. No treatment-related effects in tissues at necropsy.	6314-95-0091-TX-001
Rat, albino (5 m, 5 f)	Acute inhalation	EPA FIFRA, subdivision F, §81-3., 4 h exposure ITU/mg not recorded*.	LC ₅₀ >2.84 mg/l. No mortality but activity decrease, crust around eyes and nose, and piloerection on day of exposure. Asymptomatic by day 1 and body weight unaffected by exposure.	1723-94
Rabbit, albino (3 m, 3 f)	Skin irritation	EPA FIFRA, Subdivision F, § 81-5 ITU/mg not recorded*.	Well-defined erythema in all at site of application at 30 to 60 min, very slight to well-defined erythema in all at days 1 to 4, very slight erythema in one rabbit on day 10 and in two rabbits on day 14. No oedema at site of application. Classified as non-irritant.	6314-95-0093-TX-001
Rabbit, albino (3 m, 3 f)	Eye irritation	EPA FIFRA, Subdivision F §81-4 ITU/mg not recorded*.	In unwashed group corneal opacity in one rabbit at 24-h, no iridal effects. No corneal and iridal effects in washed group. Conjunctival redness, chemois and discharge at 1-h in both groups. Minimal conjunctival redness persisted in one rabbit in each group through day 4 but not present on day 7. Classified as non-irritant.	6314-95-0092-TX-001

* Doses based on spore count or mg product.

Table A. Toxicology profile of Bti strain AM65-52 technical material, based on acute toxicity, irritation and sensitization.

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Guinea pig (young adult m, f)	Skin sensitization	EPA FIFRA Subdiv. F §81-6, 40 CFR 152-36 ITU/mg not recorded*.	Technical material 50% w/v in water for induction and 5% w/v in water for primary challenge produced dermal sensitization. Technical material 0.5% w/v in water for re-challenge did not elicit sensitization responses.	94-8488-21

Table B. Additional toxicology profile of Bti AM65-52 technical material, based on single administration.

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Rat, Sprague-Dawley (21 m, 21 f)	Acute oral toxicity & pathogenicity	EPA FIFRA 40 CFR 158-740. Single oral dose of approximately 10^8 CFU. ITU/mg not recorded*.	No mortality, nor significant signs of toxicity, nor evidence of pathogenicity. Ruffled coat in all treated animals early in study and enlarged lungs (day 4) in treated animals. Bti found in lungs of only one treated animal on day 4. In all treated animals, total clearance occurred on day 8, with exception of faeces, which cleared on day 22. Non-toxic, non-pathogenic to rat.	G-7264.222
Rat, Sprague-Dawley (24 m, 24 f)	Acute intravenous toxicity & pathogenicity	EPA FIFRA 40 CFR 158-740. Single intravenous injection of approximately 10^7 CFU ITU/mg not recorded*.	No mortality in 22- or 50-day test periods. No treatment-related toxicity or pathogenicity. Test microbe present at high levels in spleen and liver, low in other tissues and blood. Total clearance occurred from brain, blood and caecum contents but not from lungs, spleen, liver, lymph nodes and kidney. In uncleared tissues, microbial counts remained unchanged at test day 50.	G-7264.224

* Doses based on spore count or mg product.

* Doses based on spore count or mg product.

Table B. Additional toxicology profile of Bti AM65-52 technical material, based on single administration.

Species	Test	Duration and conditions or guideline adopted	Result	Reference
Mouse, (5 m, 5 f per treatment)	Intraperitoneal injection test	EPA FIFRA 40 CFR 180-1011. 0.5, 0.05 or 0.005 mg technical material per animal (corresponding to 10^8 , 10^7 and 10^6 CFU/animal), injected in peritoneal cavity. Bti A65-52 potency 6600 ITU/mg.	No signs of toxicity during the 7-day test period.	VTP/TE-05
Rat, Sprague-Dawley (24 m, 24 f)	intra-tracheal instillation	EPA FIFRA 40 CFR 158.740. Single intra-tracheal instillation of 10^8 CFU. ITU/mg not recorded*.	No mortality in 50-day test period. Treatment-related toxicity apparent early in study with regard to respiration, locomotion, body position and coat appearance. No pathogenicity. Persistent pulmonary lesions considered not due to infectious process but presence of very numerous foreign particles in the lungs. Bti present in all tissues of treated animals, except brain. On day 50, total clearance was observed in blood, kidney and lymph nodes. Total clearance not observed by day 50 from spleen, liver, lungs and caecum contents but microbial count was substantially reduced compared with peak levels, except in spleen. Bti did not proliferate. At necropsy on days 4, 8, 15, 22, 36 and 50, pathological observations of all animals were normal.	G-7264.225

* Doses based on spore count or mg product.

Table C. Ecotoxicology profile of Bti AM65-52 technical material.

Species	Test	Duration and conditions	Result	Reference
<i>Daphnia magna</i> (water flea)	Acute toxicity	Duration: 10 days. Three replicate exposure vessels containing 10 daphnids each (30 per concentration and control). Test solutions renewed every 48 hours. ITU/mg not recorded*.	EC ₅₀ >50 mg/l Following day 10 of exposure, 93% survival at 50 mg/l, compared with 97% survival in untreated controls.	2439.6137
Chironomid midge larvae, species not stated	Mesocosm tests	Bti A65-52 WG applied on 2 occasions at 1, 9, 22.5, 45 or 90 kg/ha. ITU/mg not recorded*.	Adverse effects at ≥45 kg/ha were transient and recovery occurred 14-32 d after application	Liber K. <i>et al.</i> 1998
Bluegill sunfish (<i>Lepomis macrochirus</i>)	30-day static renewal test	Food mixed with Bti AM65-52 at nominal 500 times expected environmental concentration (3.89×10^{10} CFU/g food). 6600 ITU/mg.	No abnormal behaviour. No lesions, necroses or tumours attributable to Bti AM65-52. No adverse effects on survival, growth. Non-infective, non-pathogenic.	90-2-3228
Rainbow trout (<i>Oncorhynchus mykiss</i>)	32-day static renewal test	1.945 g test material (3.89×10^{11} CFU) suspended in 100 ml water added to 49.9 l soft reconstituted water and added to 3 test aquaria. Commercially-prepared fish food provided once daily at growth-promoting rate of 4.5% body weight. Food mixed with Bti AM65-52 at nominal 500 times expected environmental concentration (3.89×10^{10} CFU/g food). 6600 ITU/mg.	No adverse effect on survival, nor any evidence of infectivity or pathogenicity in the form of lesions, tumours or necrosis.	90-2-3242
Sheepshead minnow (<i>Cyprinodon variegates</i>)	30-day static renewal test	Nominal aqueous and dietary concentrations equivalent to 100 times and 500 times expected environmental concentration, respectively. 6600 ITU/mg.	No evidence of infectivity or pathogenicity and no adverse affect on fish growth.	90-4-3288
<i>Apis mellifera</i> (honey bee)	Oral toxicity	Bti AM65-52 suspended in water/honey, 1:1 v/v. Treatments 24 g/acre (0.1 x field rate) to 2400 g/acre (10 x field rate), duration 14 days. ITU/mg not recorded*.	Non-toxic as stomach poison to adult worker honey bees at 0.5 to 10 times the field dosage for insect control.	BATFT/C 90-833-F/C

* Doses based on spore count or mg product.

Table C. Ecotoxicology profile of Bti AM65-52 technical material.

Species	Test	Duration and conditions	Result	Reference
Mallard duck (<i>Anas platyrhynchos</i>)	Oral toxicity	Oral gavage at 3077 mg/kg ($3.4-6.2 \times 10^{11}$ CFU/kg/day for 5 days). ITU/mg not recorded*.	TEL >3077 mg/kg bw No mortality, normal appearance and behaviour. No effect on body weight or feed consumption. No pathogenicity, toxicity, or effect on survival of young mallards	161-115
Northern bobwhite quail (<i>Colinus virginianus</i>)	Oral toxicity	Oral gavage 3077 mg/kg bw/d, for 5 days 6600 ITU/mg, 2.0×10^{11} CFU/g	No pathogenicity, toxicity or effect on survival of young birds. LD ₅₀ >3077 mg/kg (equivalent to >1874 mg/kg bw/d) NOEC = 3077 mg/kg (equivalent to 1874 mg/kg bw/d)	161-114

ANNEX 2. REFERENCES

Valent Biosciences document number or other reference	Year and title of report or publication details
161-114	1990. VectoBac technical material (<i>Bacillus thuringiensis</i> var <i>israelensis</i>): An avian oral toxicity and pathogenicity study in the bobwhite.
161-115	1990. VectoBac technical material (<i>Bacillus thuringiensis</i> var <i>israelensis</i>): An avian oral toxicity and pathogenicity study in the mallard.
2439.6137	1999. VectoBac TP (ABG-6164S) – toxicity to water fleas (<i>Daphnia magna</i>) under static-renewal conditions.
90-2-3228	1990. VectoBac technical material (<i>Bacillus thuringiensis</i> var. <i>israelensis</i>) – infectivity and pathogenicity to bluegill sunfish (<i>Lepomis macrochirus</i>) during a 30-day static renewal test.
90-2-3242	1990. VectoBac technical material (<i>Bacillus thuringiensis</i> var. <i>israelensis</i>) – infectivity and pathogenicity to rainbow trout (<i>Oncorhynchus mykiss</i>) during a 32-day static renewal test.
90-4-3288	1990. VectoBac technical material (<i>Bacillus thuringiensis</i> var. <i>israelensis</i>) – infectivity and pathogenicity to sheepshead minnow (<i>Cyprinodon variegatus</i>) during a 30-day static renewal test.
BATFT/C 90-833-F/C	1990. Bee adult toxicity feeding test/chronic evaluating the comparative chronic stomach poison toxicity of <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> (<i>Bt</i>) to honey bee worker adults.
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G-7264.222	1990. Acute oral toxicity/pathogenicity study of Vectobac technical material (<i>Bacillus thuringiensis</i> var. <i>israelensis</i>) in rats.
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Golberg & Margalit 1977	Golberg, L.J. and Margalit, J., 1977. A bacterial spore demonstrating rapid larvicidal activity against <i>Anopheles sergentii</i> , <i>Uranotaenia unguiculata</i> , <i>Culex univittatus</i> , <i>Aedes aegypti</i> and <i>Culex pipiens</i> . <i>Mosq. News</i> , 37 , 355-358.
IPCS, 1999	Environmental Health Criteria, No 217. <i>Bacillus thuringiensis</i> , 1999, 105pp.
Liber <i>et al.</i> , 1998	Liber, K., Schmude, K.L. and Rau, D.M., 1998. Toxicity of <i>Bacillus thuringiensis</i> var. <i>israelensis</i> to chironomids in pond mesocosms. <i>Ecotoxicology</i> , 7 : 343-354.
US EPA, 1998	Reregistration Eligibility Decision - <i>Bacillus thuringiensis</i> - EPA738-R98-004, 157pp.
VTP/TE-05	1990. Intraperitoneal injection test with Vectobac technical powder.
WHO, 2002	The WHO recommended classification of pesticides by hazard and guidelines to classification 2000-2002, document WHO/PCS/01.5. WHO, Geneva, 2002.