Renewal
Assessment Report

Bacillus thuringiensis ssp. aizawai strain ABTS-1857

Volume 3 – B.5 Analytical methods

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
September 2018	

Table of contents

B Summary of the data and information

B.5 Analytical methods

B.5.1 Methods for the analysis of the micro-organism as manufactured

B.5.1.1 Methods for the identification of the micro-organism

Genomotyping which makes analysis of bacteria by comparison of their genomes using microarrays (van der Vossen et al. 2008 in B.5.1.2) and a rapid quantitative discriminatory PCR method (van der Vossen et al.2015 in B.5.1.2) allow unequivocal identification.

B.5.1.2 Methods for providing information on possible variability of seed stock/active micro-organism

Methods to detect spontaneous change in the seed stock/active micro-organism include monitoring growth characteristics on agar media, microscopical observations, biochemical analyses and plasmid profiles were presented in the DAR (2007), Annex C; Confidential Information C1.2.6, Smith (1990) and Benson (2005). A brief summary of each method is provided below.

Report:	IIM 1.3.3/01. Smith, R.A. (1990) (This report was previously considered in the DAR 2007.)
Title:	Microbiological characterisation of ABG-6305 production strains.
Document No:	Unpublished report No.: 1859-91Y
Guidelines:	Not stated
GLP	Yes

Abstract:
The methods used to characterise *Bacillus thuringiensis* subsp. *aizawa*i, strain ABTS-1857, ATCC-SD-1372 are described in this report. These include biochemical and morphological testing, flagellar antigen serotyping, antibiotic sensitivity pattern, characterisation of crystal protein and plasmid profile.

Conclusion:
The study describes methods suitable for the characterisation of *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857, ATCC-SD-1372.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report: R.A. Smith (1990) Microbiological Characterisation of ABG-6305 production strains. Abbot Laboratories, Unpublished report No.: 1859-91Y

GLP: Yes

Methods: The methods used to characterise *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857, ATCC-SD-1372 are described in this report and are summarised below. The methods were used to compare AGB-6305 production strain with 3 other closely related strains:

Btk-HD-1, ATCC-SD-1275 (Used in the production of DiPel®)

ABTS-26, HD-133 (an AIZ type strain from the U.S. Dept of Agriculture).

ABTS-1883, HD-11 (an AIZ type strain for serotyping from the U.S. Dept of Agriculture).

Biochemical and morphological testing

Biological and morphological characterisation was determined according to methods prescribed in Bergey's Manual of Systematic Bacteriology, Vol 2 [2,19]

Flagellar antigen serotyping

Antisera were prepared by injecting rabbits with formalin treated cell suspensions of each strain of *Bacillus thuringiensis*. Cross reaction of the antiserum produced was assessed to standard flagella antibody types for *kurstaki* (type 3a3b, antiserum #3363) and *aizawai* (type H-7, antiserum #3367) as measured by precipitation reaction.

Antibiotic sensitivity pattern

The susceptibility of the above *Bacillus thuringiensis* strains to 10 anti-microbial agents (gentamicin, kanamycin, erythromycin, clindamycin, penicillin, ampicillin, cephalothin, vancomycin, chloramphenicol, trimethoprim sulfamethoxazole) was determined using standard methodology (S.O.P 047T-12-043A; National Committee for Clinical Laboratory Standards, 1984 [7,8]). *Staphylococcus aureus*, ATCC 25923 was also included in the test to verify the test procedure.

Characterisation of crystal protein

Toxin protein crystals were extracted and purified from sporylated cultured of the organism using density gradient centrifugation. Isolated crystal proteins were dialysed overnight against deionised water and lyophilised. The isolated proteins were then analysed by SDS-PAGE in accordance with BOP 91Y-023-05 as described in section 4.3.1.

Plasmid profile

Plasmid DNA was extracted and isolated by growing cell in liquid culture and harvesting and washing the cells by centrifugation. Cell were then lysed and chromosomal DNA and cell debris separated by centrifugation. A portion of the supernatant containing the plasmid DNA was removed and the DNA precipitated by adding sodium acetate and ethanol followed by incubation at –20°C for 18 hours. The DNA pellet was dried, before re-suspending in buffer and incubating with RNase A and T1 RNase for 1 hour. Proteinase K was then added and the solution incubated for a further 2 hours. Protein was then removed from the solution by washing with buffered phenol followed by washes with tris buffer to reduce the pH of the aqueous phase to 7.6 and partitions with chloroform / isoamyl alcohol (24:1, v/v). The aqueous phase was then taken and the DNA precipitated as before by the addition of sodium acetate and ethanol and incubation at –20°C for 1 hour. Supernatant was poured off and DNA pellet dried under vacuum at room temperature. The DNA was finally re-suspended in TES buffer in preparation for analysis by gel electrophoresis.

Electrophoresis was run on Agarose gels (0.8%) made in XBE buffer and were run on a pulse wave switcher at 4°C initially for 1 hour at 80 V (9 seconds forward, 27 seconds reverse) followed by 26 hours at 50 V (60 seconds forward, 180 seconds reverse). Gels were stained with 0.6% ethidium bromide for 30 minutes and destained for 15 minutes in water. Gels were photographed under UV light.

Findings:

Biochemical characterisation

The biochemical response of the 3 organisms was very similar and majority of the response were similar to those expected by the Bergey strain type. There were minor metabolic differences, and minor difference in growth/no-growth breakpoints at various temperatures. The differences do not appear to be significant enough to identify the individual stains.

Flagellar antigen serotyping

The flagellar antigen of ABTS-1857 reacted strongly to flagellar Ab type H-7 antiserum but did not react to the flagellar Ab type 3a3b. The known H-7 serotype stains ABTS-26 and ABTS-1883 also reacted to the flagellar Ab type H-7 antiserum but did not react to the flagellar Ab type 3a3b. The BTk, HD-1 strain reacted to the flagellar Ab type 3a3b antiserum, but not to the flagellar Ab type H-7 antiserum. The result confirms the strain as flagellar serotype H-7.

Antibiotic sensitivity pattern for ABTS-1857

The sensitivity pattern for ABTS-1857 was very similar to both the other *Bacillus thuringiensis* subsp. *aizawai* strains and to the *Bacillus thuringiensis* subsp. *kurstaki*. All were susceptible to gentamicin, kanamycin, erythromycin, clindamycin, vancomycin, chloramphenicol, trimethoprim sulfamethoxazole but appear to be resistant to penicillin, ampicillin and cephalothin.

Insecticidal proteins produced by ABG-6305 strain ABTS-1857

SDS-soluble protein purified from ABTS-1857 contained 1 major protein band corresponding to a molecular mass of approximately 135 kDa. The same major protein band was also present in the other 2 *Bacillus thuringiensis* subsp. *aizawai* strains (ABTS-26 and ABTS-1883). The isolated protein from the *kurstaki* strain (Btk-HD-1) contained the same protein but also contained a second prominent band. SDS soluble protein extracted from sporylated culture of ABTS-1857 also showed a single main protein band at 135 kDa. Protein from five lots of ABTS-1857 technical powder was also isolated and all showed the major 135 kDa protein band and some other minor bands probably from other cellular proteins or components of the fermentation medium.

Plasmid profile

The profile of the plasmid DNA from ABTS-1857 was compared to the profile from 2 *Bacillus thuringiensis* subsp. *aizawai* strains (ABTS-26 and ABTS-1883) and the *kurstaki* strain (Btk-HD-1). For the ABTS-1857-strain 5 plasmid bands migrated slower than the chromosomal DNA and 10 plasmid bands migrated faster than the chromosomal DNA.

Conclusion: **The study describes methods suitable for the characterisation of *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857, ATCC-SD-1372**

Report:	IIM 1.3.3/02. Benson, T. (2005) (This report was previously considered in the DAR 2007.)
Title:	Summary Report: Genetic comparison of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 to other Bacillus strains using AFLP
Document No:	Unpublished report No.: Not available
Guidelines:	Not stated
GLP	Yes

Abstract:

Amplified fragment length polymorphism (AFLP) is used to investigate genetic differences between individuals, populations and closely related species. This is a specialist technique, which involves isolating DNA from the organism and digesting with 2 different restriction enzymes EcoRI and MseI. The resulting DNA fragments are tagged with short synthetic pieces of double stranded DNA called adapters and the tagged fragments are replicated or amplified using Polymerase Chain Reaction (PCR) using DNA primers homologous to the adaptors. A small amount of this amplified DNA if further selectively amplified by using modified primers to reduce the number of fragments that are amplified. One of the primers used in this reaction is also fluorescently labelled which will be used to visualise the resulting amplified fragments. The final amplified products are mixed with DNA size markers and separated using sequencing gel electrophoresis and the resulting gel is visualised on an automated fluorescent sequencer. Computer software is used to analyse the size of the various fragments. Each fluorescent labelling is conducted in triplicate and only fragments containing 100 to 500 base pairs which exhibiting a fluorescence of above 50 units on all three runs are included in the fingerprint. The fingerprint is presented as an electropherogram with the peak location dependent on the fragment size and the peak size representative of the number of fragments of a given size. The fingerprints are compared using a clustering algorithm to determine common peaks.

Conclusion:

AFLP provides a suitable way of genetically differentiating between the strains of Bacillus. *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857 can be clearly differentiated from the main group of pathogenic and toxigenic Bacillus strains.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report:	T. Benson, (2005) Summary Report: Genetic comparison of *Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857 to other *Bacillus* strains using AFLP. Valent Biosciences Corporation, Unpublished report No.: Not available
GLP:	Yes
Methods:	Amplified fragment length polymorphism (AFLP) is used to investigate genetic differences between individuals, populations and closely related species. This is a specialist technique, which involves isolating DNA from the organism and digesting with 2 different restriction enzymes EcoRI and MseI. The resulting DNA fragments are tagged with short synthetic pieces of double stranded DNA called adapters and the tagged fragments are replicated or amplified using Polymerase Chain Reaction (PCR) using DNA primers homologous to the adaptors. A small amount of this amplified DNA if further selectively amplified by using modified primers to reduce the number of fragments that are amplified. One of the primers used in this reaction is also fluorescently labelled which will be used to visualise the resulting amplified fragments. The final amplified products are mixed with DNA size markers and separated using sequencing gel electrophoresis and the resulting gel is visualised on an automated fluorescent sequencer. With each set of reactions an AFLP reaction containing *Bacillus anthracis* Vollum DNA as a reference to allow comparisons between different runs and different gels. Computer software is used to analyse the size of the various fragment. Each fluorescent labelling is conducted in triplicate and only fragments containing 100 to 500 base pairs which exhibiting a fluorescence of above 50 units on all three runs are included in the fingerprint. The fingerprint is presented as an electropherogram with the peak location dependent on the fragment size and the peak size representative of the number of fragments of a given size. The fingerprints are compared using a clustering algorithm to determine common peaks, which are then assigned average peak values and the top 40 peaks used to calculate the Jaccard coefficient. Dendrograms were produced using the similarity matrix of Jaccard coefficient and the unweighted pair group mean average method.
Findings:	Based on the fingerprint electopherograms for Bacillus thuringiensis subsp. aizawai, strain ABTS-1857, the organism has been placed in the phylogenetic dendrogram at cluster 1 Branch C with many other Bt strains which are not toxigenic to vertebrates. It also shows that Bacillus thuringiensis subsp. aizawai, strain ABTS-1857 is distinct from the pathogenic and toxigenic Bacillus isolates which are grouped in Cluster 2 Branch F.
Conclusion:	AFLP provides a suitable way of genetically differentiating between the strains of Bacillus. Bacillus thuringiensis subsp. aizawai, strain ABTS-1857 can be clearly differentiated from the main group of pathogenic and toxigenic Bacillus strains.

For renewal of the strain two new studies are submitted describing the methods of strain specific markers for unequivocal identification of a number of Bacillus species to subspecies and strain level. The first study is about genomotyping which makes analysis of bacteria by comparison of their ge-

nomes using microarrays. The second study is about a rapid quantitative discriminatory PCR method for detection of Bacillus thuringiensis subsp. strains. Both studies are summarised below.

Data point addressed:	KMA 1.3/01, KMA 4.1.1/01
Author(s) (year):	van der Vossen *et al*. (2008)
Title:	Strain identification of Valent BioSciences Corp.'s strain collection.
Laboratory report / project number:	V8278
Testing facility:	TNO (The Netherlands)
Published:	No
Test guideline used:	Not stated
Deviations:	Not stated
GLP:	No

Objective: The objective of the study was to identify a number of *Bacillus* species to subspecies and strain level using strains developed for commercial application by Valent BioSciences Corporation (VBC) as well as *Bt* strains collected in the field.

Methods: Genomotyping describes the analysis of bacteria by comparison of their genomes using microarrays. This method allows for comprehensive DNA and RNA analyses to characterize genetic diversity and gene expression in a genome-wide manner. 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. The methods involve fluorescent labelling the DNA to be probed and the results then present differential fluorescence intensities for the reference and sample genome for each gene. The log-ratio of these intensities is used for classifying each gene of the sample genome for absence or presence with respect to the reference genome. Included in genomotyping were strains developed for commercial application by VBC as well as Bt strains collected in the field. The DNA isolated was fluorescently labelled; Cy5 dye was incorporated into the DNA of the tester strains as well as pure cultures from the laboratory's culture collection. Cy3 dye was incorporated in a mixture of isolated DNA from different strains from the *B. cereus* group to act as an internal reference. The internal reference was a mix of *B. cereus, B. thuringiensis* and *B. weihenstephanensis* strains. Using two dyes improves the robustness of the hybridization test. Gene complement differences can then immediately be registered and quantified.

Results: Identification of the applicant's strain ABTS-1857 was accomplished by separating out the applicant's specific strains from other strains of the same Bt subspecies in combination with further differentiation of the different strains within the subspecies clusters. By repeating the methodology in duplicate/triplicate the robustness and reproducibility of the genomotyping method is demonstrated. The genomotyping technology is shown to be the methodology of choice to discriminate closely related strains. Gene complement differences are observed between the different subspecies and can also be observed even between strains of the same subspecies. Identification to subspecies and strain level was accomplished.

A second study of analysis to unequivocally identify the organisms down to strain level is submitted.

Data point addressed:	KMA1.3/02, KMA 4.1.1/02 **Confidential information, Valent Biosciences Corp. Trade Secret**.
Author(s) (year):	van der Vossen et al. (2015)
Title:	A rapid quantitative discriminatory PCR method for detection of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, *Bacillus thuringiensis* subsp. *aizawai* strain ABTS1 857 and *Bacillus thuringiensis* subsp. *israelensis* AM65-52 in a possible background of *Bacillus cereus* group species

Laboratory report / project number:	R10428
Testing facility:	TNO (The Netherlands)
Published:	No
Test guideline used:	Not stated
Deviations:	Not stated
GLP:	No

A second study of analysis to unequivocally identify the organisms down to strain level is submitted. This demonstrates a more user-friendly discriminatory test based on multiplex real-time quantitative PCR (qPCR) for the quantitative discriminatory detection of Bacillus thuringiensis subsp.kurstaki (Btk) strain HD-1, Bacillus thuringiensis subsp. aizawai strain 1857 (Bta) and Bacillus thuringiensis subsp. israelensis AM65-52 (Bti) that may be present among other Bacillus cereus group species in food crops and surface water.

Objective: To differentiate between the MPCA strains that may be present among other *Bacillus cereus* group species in food crops and surface water.

Methods: *Bacillus cereus* produces different toxins to the MPCA BTk ABTS-351. The strains cannot be distinguished using traditional microbial plating methods used in safety testing facilities in the food industry. This newly described method builds on previous studies commissioned by the applicant such as the report of van der Vossen *et al*., 2008 (ref. V8278; KMA 1.3/01) described above to simplify and develop the discriminatory techniques into a rapid identification system using modern multiplex real-time quantitative PCR (qPCR) technology. This enables differentiation of the MPCA strains that may be present among other *Bacillus cereus* group species in food crops and surface water. The method uses genomotyping data gained from the applicant's research studies to support this renewal evaluation, specifically, sequenced differential probes, bespoke designed PCR priming sequences and VBC strain genome sequencing.

Results: The results of the method development showed that *Bt kurstaki* can be quantitatively detected in PCR using the highly specific BTK-B10 primer/probe set. The primer/probe set BTK-B10 is most specific for HD-1 and detects also two other *Bt kurstaki* strains targeting a specific plasmid sequence and does not detect other *Bacillus cereus* group strains. For the *Bacillus cereus* species group, a generic qPCR was developed using primer/probe set B.CTW-C01. All *Bacillus cereus* group strains are detected with one *Bacillus thuringiensis* exception. The conclusions of the study demonstrate that qPCR detections are functional and fully culture independent. Detection is quantitative and reflects CFU counts. Detection functions in multiplex qPCR, multiplex qPCR protocols for the detection of *Bacillus cereus* and *Bacillus thuringiensis* in lettuce are functional, detection limits are approximately within $1x10^2$ to $1x10^3$ spores per gram lettuce produce and multiplex qPCR protocols are executable within eight hours.

Bta strain ABTS 1857 can be quantitatively detected in qPCR using the BIA-BIO primer/probe set

- Primer/probe set BTA-B10 is specific for Bta strain ABTS 1857 and also detects six other Bta strains and Bt strain 05.0660
- Primer/probe set BTA-B10 does detect a few other Bc group strains at high Ct values but these signals are negligible in terms of false positives
- Primer/probe set BTA-B10 is based on the BTK-B10 set with minor modifications that make it specific for Bta; it does not detect any Btk strains

In addition, the applicant performed an annotation against completed genomes of Bacillus cereus and three Valent strains of Bacillus thuringiensis, see below.

Data point addressed:	KMA 1.3/03
Author(s) (year):	Anon. (2015) (a) & (b)
Title:	Annotation of three strains of Bacillus thuringiensis Bta, Bti and Btk. A Powerpoint presentation and supporting graphics.
Laboratory report / project number:	Not applicable – PowerPoint presentation
Testing facility:	Not applicable
Published:	N
Test guideline used:	N/A
Deviations:	N/A
GLP:	N

Summary

Annotation was performed against completed genomes of Bacillus cereus and three Valent strains of Bacillus thuringiensis. Toxin related information was considered; Two protein complexes (hemolysin BL → hbl operon and proteins) and non-hemolytic enterotoxin → nhe operon and proteins). Also one enterotoxic protein was considered (cytotoxin K → cytK gene and protein) and emetic toxin (cereulide → ces gene and protein).

hemolysin BL; hbl operon; Not found in all Bacillus cereus and Bacillus thuringiensis strains. In annotated genomes sometimes only genes are found, not full operon. In visualization of the operon, the first sequence is Bacillus thuringiensis subsp. israelensis, others are completed genomes.

Conclusions concerning hbl; The hbl operon is present in Bti including all genes. The hbl operon is fully absent in Bta. A part of the operon is present in Btk which does only contain the lytic compont genes L1 and L2.

Non-hemolytic enterotoxin; nhe operon; Found in all species. Seems to be active in both Bacillus cereus and Bacillus thuringiensis.

Conclusion concerning nhe; All three strains Bta, Bti and Btk do contain the full nhe operon.

Conclusions regarding cytK; The cytK gene is present in all three strains Bta ABTS-1857, Bti AM65-52 and Btk ABTS-351. The cytK genes of the three Bt strains are extended at the 5'prime end of the gene, which implies also a stretch of an additional 32 amino acids at the N-terminus of the enterotoxic protein.

The CytK gene found is not the CytK-1 gene which is considered more virulent.

Conclusions regarding ces; The ces gene has not been found in the genome sequence of the three Bt strains.

B.5.1.3 Methods to differentiate a mutant of the micro-organism from the parent wild strain

See B.5.1.2

B.5.1.4 Methods for the establishment of purity of seed stock from which batches are produced and methods to control that purity

See C.1.1.2

B.5.1.5 Methods to determine the content of the micro-organism in the manufactured material used for the production of formulated products and methods to show that contaminating micro-organisms are controlled to an acceptable level

The activity of the Bta products essentially depends on the content of the crystal proteins, in particular the Cry1Ab protein. Several methods have been used to quantify Cry1Ab including enzyme linked immunosorbent assay (ELISA), sodium dodecyl sulfate polyacrylamide gel electrophoresis densiometry (SDS-PAGE) and total protein assays such as the Bradford assay.

Measurements of the crystal protein content and measurements of insecticidal potency were initially described in the DAR (2007); Annex C, Volume 4, C1.1.3. A brief summary of each method is provided below;

SDS-PAGE

Report:	IIM 1.4.1/01, Short, M. (1990) (This report was previously considered in the DAR 2007.)
Title:	Quantification of the active ingredient ABG-6305 technical powder by SDS-PAGE
Document No.	Abbot Laboratories, Unpublished report No.: 45-255-62
Guidelines:	US EPA OPPTS 885.1400 and 885.1500
GLP:	Yes

Abstract:

A method has been developed using SDS-PAGE to quantify the toxic protein (active ingredient) in the manufactured products of *Bacillus thuringiensis* var. *aizawai* (ABTS 1857).

Sample preparation - Triplicate 100 mg samples of the technical powder were homogenised by hand in crystal dissolution buffer and sonicated in an ultrasonic bath for 2 minutes. The suspension was then heated for 20 minutes at 70°C and 1 mL of each suspension was transferred to 9 mL of final buffer solution in to screw top test tubes. The tubes were sealed and heated at 100°C for 5 minutes, allowed to cool, and mixed by inverting. A 0.5 mL aliquot of each sample was combined with 0.5 mL of 60% glycerine solution, mixed and centrifuged at 13000 rpm for 1 minute prior to application of the sample to the SDS-PAGE gel.

Preparation of 135 kDa protein standards – The protein standard was thawed and combined with final sample buffer solution and 60% glycerol solution. Standards of 0.055, 0.11 and 0.22 mg/mL were prepared. The standards were prepared in screw top test tubes which were sealed and heated at 100°C for 5 minutes, cooled, centrifuged at 1000-2000 rpm prior to application to the gel.

Running of the gels – Each gel is loaded with two wells containing the 0.055 mg/mL standard, one well containing the 0.11 mg/mL standard and two wells containing the 0.22 mg/mL standard. The two outermost wells were filled with blank sample or standard and all other wells were used for samples. The gel is connected the power supply and run at a constant 100 V to move the samples into the stacking gel. Once the samples had moved into the stacking gel then the constant voltage was increased to

200 V for the duration of the run. After the run the gel was removed from the gel slab device and transferred to the staining solution for 15 minutes. The gel was then de-stained and scanned using a densitometry to determine the area of the peaks corresponding to the 135 kDa protein. The areas of the standards were used to construct a calibration curve from which the concentration of the samples could be determined.

Conclusions:

The SDS-PAGE method is suitable for the determination of the weight of 135 kDa protein toxin (the active ingredient) in the ABG-6305 technical powder, although the method has not been fully validated and a data gap was identified in the EFSA Conclusions[1]. Further validation is therefore necessary.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report:	M. Short (1990) Quantification of the active ingredient ABG-6305 technical powder by SDS-PAGE. Abbot Laboratories, Unpublished report No.: 45-255-62
GLP:	Yes
Specificity:	Determination of 135 kDa protein toxin in ABG-6305 technical powder
Method:	Sample preparation - Triplicate 100 mg samples of the technical powder were homogenised by hand in crystal dissolution buffer and sonicated in an ultrasonic bath for 2 minutes. The suspension was then heated for 20 at 70°C and 1 mL of each suspension was transferred to 9 mL of final buffer solution in to screw top test tubes. The tubes were sealed and heated at 100°C for 5 minutes, allowed to cool, and mixed by inverting. A 0.5 mL aliquot of each sample was combined with 0.5 mL of 60% glycerine solution, mixed and centrifuged at 13000 rpm for 1 minute prior to application of the sample to the SDS-PAGE gel. Preparation of 135 kDa protein standards – The protein standard was thawed and combined with final sample buffer solution and 60% glycerol solution. Standards of 0.055, 0.11 and 0.22 mg/mL were prepared. The standards were prepared in screw top test tubes which were sealed and heated at 100°C for 5 minutes, cooled, centrifuged at 1000-2000 rpm prior to application to the gel. Running of the gels – Each gel is loaded with 2 wells containing the 0.055 mg/mL standard, 1 well containing the 0.11 mg/mL standard and 2 wells containing the 0.22 mg/mL. The two outermost well were filled with blank sample or standard and all other wells were used for samples. The gel is connected the power supply and run at a constant 100 V to move the samples into the stacking gel. Once the samples had moved into the stacking gel then the constant voltage was increased to 200 V for the duration of the run. After the run the gel was removed from the gel slab device and transferred to the staining solution for 15 minutes. The gel was then de-stained and scanned using a densitometry to determine the area of the peaks corresponding to the 135 kDa protein. The areas of the standards were used to construct a calibration curve from which the concentration of the samples could be determined.
Findings:	Correlation curves from two sets of standards on respective gels showed correlation coefficients of >0.993. There is no further validation on the method. The method has however been used to measure the weight of the 135 kDa protein toxin in five lots of ABG-6305 technical powder.
Conclusions:	**The SDS-PAGE method is suitable for the determination of the weight of 135 kDa protein toxin (the active ingredient) in the ABG-6305 technical powder, although the method has not been fully validated.**

A further report was described in the DAR (2007); Annex C, Volume 4, C1.1.3; Anon (2000) detailing a bioassay with moth larvae. A similar bioassay has been described and validated in B.5 MP, Section 5.1.1, Wicker (2016).

Further Validation

Since the preparation of the DAR (2007), a published paper is now available comparing and validating methods used to quantify the Cry1Ab toxin. A full summary is provided below.

Report:	KMA 4.1.5/01, Crespo, A.L.B et al (2008)
Title:	Comparison and validation of methods to quantify Cry1Ab toxin from *Bacillus thuringiensis* for standardization of insect bioassays
Document No.	Applied and Environmental Microbiology, Jan 2008, p 130-135, Vol. 74, No. 1
Guidelines:	Not stated
GLP:	No

Principle of Method:

Bradford assay: The concentration of Cry1Ab in the unknowns was determined by preparing a range of standard concentrations that provides a linear response between 0 and 600 µg of protein/mL. Bovine serum albumin (BSA), standard grade at 2000 µg/mL, was diluted in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10) according to manufacturer's instructions. The concentrations of standards were 25, 125, 250 and 500 µg/mL. Absorbance readings at 595 nm were determined with a microplate reader after duplicates of standards and unknowns had been incubated for 10 min with Coomassie G-250 dye. Determinations were replicated at least four times for each preparation. The Coomassie Plus reagent was allowed to reach room temperature before incubation with standards and unknown.

ELISA: Unknowns were diluted in sample extraction dilution buffer and centrifuge tubes (1.5 mL) were used to prepare 1 mL dilutions. The Cry1Ab batches used to prepare the standards were diluted to concentrations between 0.0 to 4.0 ng/mL. The kit is provided with removable strips with eight wells each. Volumes were transferred to ELISA strips with a multichannel pipette to ensure the same incubation period in each well. Determinations were performed in two strips where a blank, four Cry1Ab standards, and three diluted Cry1Ab samples were assayed in duplicate. Determinations were replicated at least three times.

SDS-PAGE:
Bovine serum albumin (BSA) was diluted to 600, 400, 267, 178 and 119 µg/mL in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10) with phenylmethylsulfonyl fluoride at 0.5 mM to minimize protein degradation. To prevent oxidation during electrophoresis, BSA and Cry1Ab proteins were denatured by reduction-alkylation prior to quantification. Standards and samples were subjected to SDS-PAGE in 12% polyacrylamide Tris-HCl Ready Gels (Bio-Rad) for 2 h at 80 V and ambient temperature. Gels were replicated four times. After electrophoresis, gels were washed with water (30 s) and fixed with 12% trichloroacetic acid and 3.5% 5-sulfosalicyclic acid. The gels were then stained using 0.1% (wt/vol) Brilliant Blue G, 0.29M phosphoric acid and 16% saturated ammonium sulfate for 2 h and rinsed with 10% acetic acid in 25% methanol (30 s with shaking). The gels were destained and the remaining acetic acid removed by washing (three times) and soaking in methanol (up to 24 h). After destaining, the gels were photographed by using Gel Doc 2000 documentation system (Bio-Rad) and analysed densitometrically with Quality One 4.2.3 software (Bio-Rad). The stained gel is scanned and band intensity is correlated to protein quantities.

Recovery:

The determination of accuracy for the active substance in the technical material is not required according to SANCO/3030/99 rev.4.

Precision:

The precision of each quantification method was compared based on the calculated coefficient of variation for concentration estimates obtained for each Cry1Ab batch. Significant difference in Cry1Ab concentrations were detected amongst the three methods of quantification. A comparison across all toxin sources showed that the Bradford assay produced statistically higher estimates than the ELISA and SDS-PAGE methods.

In addition, these results indicate that ELISA exhibited the lowest precision among the methods tested. This is mainly due to the 200,000 fold dilution of stock solutions which is necessary to obtain concentrations within the range of the standards used to generate the curves. As a consequence, slight dilution errors may cause higher variation in the concentration estimates.

The precision values were ≤20% with the exception of the ELISA method, where the %RSD was quoted as 26.5%. From these results, both the Bradford and SDS-PAGE methods are therefore considered acceptable (RSD≤20%) according to the current guidelines, SANCO/3030/99 rev.4.

Table 4: Cry1Ab content in different sources obtained by three quantification methods

Toxin Source	Method	Cry1Ab Concentration (µg/mL) ±SE	RSD(%)
University of Nebraska	Bradford	409.8 ±6.1	4.5
	ELISA	311.4 ±29.2	26.5
	SDS-PAGE/densitometry	279.3 ±8.0	5.7
Auburn University	Bradford	315.3 ±2.6	2.4
	ELISA	293.7 ±12.8	10.6
	SDS-PAGE/densitometry	369.9 ±28.5	15.4
Monsanto Company	Bradford	365.9 ±5.3	5.4
	ELISA	352.3 ±16.0	12.8
	SDS-PAGE/densitometry	338.1 ±0.4	0.2

Specificity:

SDS-PAGE allows quantification of specific protein bands of interest. Interference from immunologically reactive Cry1Ab residues does not occur. Western blots of the different Cry1Ab batches revealed immunologically reactive peptides other than the truncated Cry1Ab protein. SDS-PAGE is specific to the Cry1Ab protein, while the ELISA method will measure the total protein content. This will include the other reactive peptides noted in the Western blot which may have contributed to the overestimation for the ELISA method.

Conclusion:

The validation above shows that quantification of Cry1Ab by SDS-PAGE is the preferred method and will improve data consistency in monitoring efforts to identify Cry1Ab. The SDS-PAGE method is specific to the 135 kDa protein toxin (the active ingredient) and has provided consistent results upon validation (%RSD ranged from 0.2-15.4%). The validation above meets the requirements of SANCO/3030/99 rev.4 in respect to specificity and precision. Further validation is not considered necessary as all techniques are widely used microbiology methods which have been used for over 20 years.

Overall Conclusion:
Sufficient methods exist for the determination of the content of *Bacillus thuringiensis* ssp. *aizawai* (strain ABTS 1857). Although the validation is limited, the techniques are widely used microbiology methods which have been used for over 20 years and are considered adequate.

.:

B.5.1.6 Methods for the determination of relevant impurities in the manufactured material

The strains may produce enterotoxins, β-exotoxin and cytolytic proteins and these may be present in the formulated product; a data gap was identified to address the validation of methods for the analysis of enterotoxins, beta-exotoxins, contaminating microorganism and parasporal proteins in the EFSA conclusions[1].

Methods for the determination of β-exotoxin by HPLC-UV (Lee, 1990) and by House Fly Bioassay (Jaronski, 1991)(Benzon 2005) were developed and validated. Both methods were submitted and described under C.1.1.2 in Annex C: Confidential Information of the DAR (2007). Further validation of the HPLC-UV method was performed prior to analysis of β-exotoxin in 3 further batches of ABG 6305 Technical Powder. β-exotoxin was not detected (<0.7ppm) (Chang, 1994). A brief description of all methods is provided below;

Report 1:	IIM 4.3.5/03. Lee, J.Y. (1990) (This report was previously considered in the DAR 2007.)
Title:	Validation of HPLC assay for β-exotoxin impurity in ABG-6305, technical powder, (Project number 45-254-62) – Addendum to MRID 41722510
Document No.	Abbot Laboratories, Unpublished report No.: 45-254-62
Guidelines:	Not stated
GLP:	Yes

Report:	IIM 4.3.5/04. Lee, J.Y. et al (1990) (This report was previously considered in the DAR 2007.)
Title:	HPLC assay for Beta-exotoxin in ABG-6305 (CenTari) technical powder – Second addendum to MRID 41722510
Document No.	Abbot Laboratories, Unpublished report No.: 60-825-62
Guidelines:	Not stated
GLP:	Yes

Abstract:

An HPLC method with UV detection has been developed for the determination for β-exotoxin impurity in ABG-6305. Samples of the technical powder are extracted with 0.05M KH_2PO_4, centrifuged and the supernatant passed through a 0.45 µm membrane with the aid of vacuum. Cleanup of the sample is achieved by passing 30 mL of the filtrate onto a pre-conditioned aminopropyl Bond-Elute® cartridge. The β-exotoxin is then eluted from the cartridge with 1N KOH, centrifuged before the immediate adjustment of the pH to 7±1 with 3 drops of 85% phosphoric acids. The extract is then diluted with distilled water, filtered and analysed by HPLC with UV detection at 260 nm.

Validation has been performed to assess the linearity, specificity, accuracy and precision of the method. Linearity of the method was confirmed over the range 0.81 to 4.84 µg/mL using six calibration points. A correlation coefficient of >0.999 was obtained. Recoveries from fortified samples at 3 levels were all within the acceptable range of 70 to110% and the mean recovery was 98.0%. Eight replicate analyses of a sample fortified at 8 µg/g were analysed using the analytical method. The mean recovery using peak area determination was 85.5% and the RSD was 11. Although the precision was only measured at one level, the RSD was <20% and therefore within acceptable limits.

Conclusion:

The HPLC method provides sensitive and reliable method of detecting and measuring β-Exotoxin in ABG-6305 technical powder. The method was also shown to be up to 50 times more sensitive than the house fly bioassay.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

J.Y. Lee (1990) Validation of HPLC assay for β-exotoxin impurity in ABG-6305, technical powder, (Project number 45-254-62) – Addendum to MRID 41722510. Abbot Laboratories, Unpublished report No.: 45-254-62 and J.Y. Lee *et al* (1990) HPLC assay for Beta-exotoxin in ABG-6305 (CenTari) technical powder – Second addendum to MRID 41722510. Abbot Laboratories, Unpublished report No.: 60-825-62	
GLP:	Yes
Specificity:	Determination of β-exotoxin in technical powder ABG-6305
Method:	Samples of the technical powder (2.5 g) are weighed into centrifuge tubes and extracted by shaking with 50 mL of 0.05M KH_2PO_4 at pH 3 for 10 minutes. For procedural recovery samples the dry samples should be fortified with standard solution prepared in 0.05M $KH2PO_4$ and the volume of extraction solvent adjusted to compensate for the addition. The sample is centrifuged at 300 rpm for 20 minutes and the supernatant passed through a 0.45 µm membrane with the aid of vacuum. Cleanup of the sample is achieved by passing 30 mL of the filtrate onto a preconditioned aminopropyl Bond-Elute® cartridge. The column is then washed with the following: 15 mL of 0.05M $KH2PO_4$, pH 3 15 mL of methanol / 0.05M $KH2PO_4$, pH 3 (1:1, v/v) 10 mL of methonal 5 mL of 0.05M $KH2PO_4$, pH 3 The β-exotoxin is then eluted from the cartridge with 2x 2mL portions of 1N KOH and each eluate centrifuged for 15 seconds at 1000 rpm before the immediate adjustment of the pH to 7±1 with 3 drops of 85% phosphoric acids. The extract is then diluted with distilled water, filtered if required and analysed by HPLC.

HPLC analysis is conducted using a Waters µ-Bondapack C18 column (30 x 0.39 cm, 10µm) eluted with 0.05 M potassium phosphate buffer with UV detection at 260 nm.

Standard solutions of are prepared in water and a standards concentrations of 0.81 to 4.84 µg/mL were prepared to determine linearity of the HPLC detector response.

Validation: Specificity: The peak was well separated from other peaks in the chromatogram. Chromatograms of unfortified ABG-6305 show no interference at the retention time of β-exotoxin.

Linearity: Calibration response curve was constructed over the range 0.81 to 4.84 µg/mL using six calibration points. The response was linear and a correlation coefficient of >0.999 was obtained. Acceptable calibration was obtained from both peak height and peak area measurements.

Accuracy: The recovery from fortified samples at 3 levels was determined all were within acceptable range of 70 to110% and the mean recovery was 98.0%. There was no replication at each of the levels measured.

Precision: Eight replicate analyses of a sample fortified at 8 µg/g were analysed using the analytical method. The mean recovery using peak area determination was 85.5% and the RSD was 11. Although the precision was only measured at one level, the RSD was <20% and therefore within acceptable limits.

Limit of Quantification: In the second addendum an assessment of the Method Detection Limit (MDL) is made based on 3x the signal noise in the chromatogram obtained from the analysis of control ABG-6305 technical powder. The calculated MDL was 1µg/g. Calculation showed that the lowest standard in the calibration curve was equivalent to a concentration of 2.7 µg/g. It was considered reasonable that measurement at 3x the baseline noise, approximately 1/3 of the lowest calibration standard could be used as the method detection limit. No fortification experiments were conducted at this level.

The second addendum report also evaluated the method detection limit for the housefly bioassay reported by Jaronski (1991), which is summarised below. In this investigation a no observed effect level from the average of 27 experiments was 0.30 µg/mL. This NOEL concentration in the test is equivalent to 50 µg/g in the ABG-6305 technical powder. Therefore the HPLC method was shown to be up to 50 times more sensitive at detecting β-Exotoxin than the housefly bioassay.

Conclusion: **The HPLC method provides sensitive and reliable method of detecting and measuring β-Exotoxin in ABG-6305 technical powder. The method was also shown to be up to 50 times more sensitive than the house fly bioassay.**

:

Report 2:	IIM 1.4.2/05. Jaronski, S.T. (1991) (This report was previously considered in the DAR 2007.)
Title:	Analysis of beta-exotoxin (thuringiensis) content of five lots of ABG-6305 technical powder by housefly bioassay.
Document No.	Abbot Laboratories, Unpublished report No.: 910-9009
Guidelines:	Not stated
GLP:	Yes

Abstract:

Three day old house fly larvae (*Musca domestica* L.) are exposed to the test substance in CSMA Fly medium for 10 days. The results of the test are measured by the percentage of the adult flies that do not emerge. The test is performed on both autoclaved and non-autoclaved test substance as non-autoclaved test substance has been shown to cause non β-exotoxin related mortalities in house fly larvae. Reference standards of β-exotoxin are also analysed in the test, both with and without autoclaving to demonstrate the effect of autoclaving on the β exotoxin. The bioassay of autoclaved and non-autoclaved test substance showed that there was an almost complete disappearance of fly larvae mortality due to autoclaving. The results therefore show that the component responsible for the fly mortality was a heat labile toxic which disappeared upon autoclaving. Based on the finding of this study no β-exotoxin was found in any of the five batches tested.

Conclusion:

The bioassay method is suitable for the determination of β-exotoxin in ABG-6305 technical material. There was no β-exotoxin found in the five batches of ABG-6305 analysed in this study.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference

S.T. Jaronski (1991) Analysis of Beta-exotoxin (thuringiensis) content of five lots of ABG-6305 technical powder by housefly bioassay. Abbot Laboratories, Unpublished report No.: 910-9009

GLP: Yes

Specificity: Detection of β-exotoxin in technical powder ABG-6305

Method: Three day old house fly larvae (*Musca domestica* L.) are exposed to the test substance in CSMA Fly medium for 10 days. The results of the test are measured by the percentage of the adult flies that do not emerge. The test is performed on both autoclaved and non-autoclaved test substance as non-autoclaved test substance has been shown to cause non β-exotoxin related mortalities in house fly larvae. Reference standards of β-exotoxin are also analysed in the test, both with and without autoclaving to demonstrate the effect of autoclaving on the β exotoxin.

A stock reference standard of the β-exotoxin was prepared by weighing 100 mg and diluting to 50 mL with deionised water. A sub-sample of this solution was autoclaved. Both autoclaved and non-autoclaved solution was diluted to a 50 µg/mL and then further dilutions were made to produce a standard curve ranging from approximately 0.4 to 4.0 µg/mL. Test substance was prepared by suspending 1.2 g in 100 mL of deionised

water. A sub-sample of this was autoclaved and then both autoclaved and non-autoclaved test substance solution was diluted 1:1 to produce a 6000 µg/mL suspension of the test substance. The test was conducted by adding 5mL of diluted test substance or reference standard or water (untreated control) to vials containing 1 teaspoon of CSMA medium (Ralston Purina). Both autoclaved and non autoclaved samples were prepared. Ten 3 day old larvae were then added to each vial. The vial was covered with paper towelling and the vial incubated at 28°C for 10 days.

After incubation the vials are opened and the number of adult flies counted. The presence of atypical larval mortality and pupa deformities, and adult deformities resulting from sub-lethal β-exotoxin exposure should be noted.

The number of emerged adult flies was subtracted from 10 to give the number of non emergents, which was averaged for replicated of a given dose. The percent mortality calculated and corrected for mortality in the untreated controls. LC_{50} for autoclaved and non-autoclaved reference standards are calculated using probit analysis, and the percent change due to autoclaving calculated. The corrected percent mortality for each autoclaved test sample was plotted on the log dose-probit regression for the corresponding reference standard to determine the theoretical β-exotoxin concentration in the test solutions. If no mortality is observed for the test substance then the concentration can be assumed to be below that of the reference standard where 0% morality was observed (LC_0, or no observed effect level).

The method was used to determine the β-exotoxin content of 5 lots of test substance.

Findings: Autoclaving of the reference standard for 30 minutes was shown to decrease the bioactivity of the β-exotoxin by 28.

8% (mean of 13 replicate experiments).

The bioassay of autoclaved and non-autoclaved test substance showed that there was an almost complete disappearance of fly larvae mortality due to autoclaving. After correction for mortality in the controls, the level of mortality from the autoclaved test substance was considered to be negligible. Mortality in the control was always less than 5%.

The results show the fly mortality in the non-autoclaved test substance was not due to β-exotoxin but due to some other heat-labile toxic component. Based on the finding of this study no β-exotoxin was found in any of the five batches tested.

Conclusions: **The bioassay method is suitable for the determination of β-exotoxin in ABG-6305 technical material. There was no β-exotoxin found in the five batches of ABG-6305 analysed in this study.**

Report 3:	IIM 1.4.2/07. Benzon, G.L. (2005) (This report was previously considered in the DAR 2007.)
Title:	Bioassays for the presence of beta-exotoxin in samples of Xentari® Technical Slurry using house fly larvae.
Document	Benzon Research Inc., Unpublished report No.: VB05016b

No.	
Guidelines:	Not stated
GLP:	Yes

Abstract:

A method for the determination of β-exotoxin in samples of Xentari® Technical Slurry was described. In this study the presence of β-exotoxin was investigated in 3 production batches of the technical slurry and two reference standards for β-exotoxin were used as positive controls. All determinations were conducted in triplicate. No validation was performed as part of the study.

Conclusion:

The study presents a method that is suitable for determining the presence of β-Exotoxin in XenTari fermentation slurry. No β-Exotoxin was detected in any of the three batches of XenTari fermentation slurry that were tested.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report: G.L. Benzon (2005) Bioassays for the presence of beta-exotoxin in samples of Xentari® Technical Slurry using house fly larvae. Benzon Research Inc., Unpublished report No.: VB05016b

GLP: No

Specificity: Detection of β-exotoxin in Xentari® technical slurry.

Method: House fly larvae (*Musca domestica*) are exposed to high concentrations of *Bacillus thuringiensis* (Bt) technical slurry prepared in house fly rearing medium. If the high concentration of Bt does not cause significant mortalities of the fly larvae, then it is assumed that there is no β-exotoxin present. Positive controls are included in the test containing the lowest concentration of that is expected to reliably kill 100% of the larvae. In this study the presence of β-exotoxin was investigated in 3 production batches of the technical slurry and two reference standards for β-exotoxin were used as positive controls. All determinations were conducted in triplicate.

The house fly bioassay is conducted in plastic cups. The house fly medium is composed of wheat bran and brewer yeast, 3:1, + 0.228% methyl paraben to control mould. Samples are prepared by autoclaving for 30 minutes, which kills any Bt spores and denatures any Bt endotoxins, but leaves the β-exotoxin unchanged. Liquid samples (1.5 mL) are mixed with 13.5 mL of water in the bioassay cup to a final volume of 15 mL. Dry samples need to be suitably diluted for the test. Positive controls are preparing a dilution of so that the concentration of β-exotoxin in the medium is 2 µg/mL. Place 12.5 g of the dry media into each cup containing the sample or standard and mix well. The fly eggs are added in a water suspension of 50 eggs/mL. While agitating the solution dispense 1 mL into each of the bioassay cups. Cover the cup with a plastic lid, which has been perforated to allow air exchange and incubate at 27-28 °C for 5 days. Count the number live larvae in each cup. If β-exotoxin is present the fly larvae will be dead, if there is no β-exotoxin present then there should be 25 to 50 live larvae.

Findings: The results of the testing are summarised in Table 4.3.5-01 and show that that the three slurry batches and the untreated control all contained living fly larvae. The two positive controls did not contain any live fly larvae. The result confirms that the three batches of XenTari fermentation slurry do not contain β-Exotoxins.

Table 4.3.5-01
Number of house fly larvae found after 5 days incubation

Sample Tested	Number of live larvae			
	Rep. 1	Rep.2	Rep.3	Mean
β-Exotoxin Type I	0	0	0	0.0
β-Exotoxin Type II	0	0	0	0.0
XenTari Fermentation Slurry run 2734	25	24	24	24.3
XenTari Fermentation Slurry run 2735	25	27	35	29.0
XenTari Fermentation Slurry run 2736	18	32	14	21.3
Untreated control	22	31	35	29.3

Conclusion: **The study presents a method that is suitable for determining the presence of β-Exotoxin in XenTari fermentation slurry. No β-Exotoxin was detected in any of the three batches of XenTari fermentation slurry that were tested.**

G.L. Benzon (2005) Bioassays for the presence of beta-exotoxin in samples of Xentari® Technical Slurry using house fly larvae. Benzon Research Inc., Unpublished report No.: VB05016b

GLP: No

Specificity: Detection of β-exotoxin in Xentari® technical slurry.

Method: House fly larvae (*Musca domestica*) are exposed to high concentrations of *Bacillus thuringiensis* (Bt) technical slurry prepared in house fly rearing medium. If the high concentration of Bt does not cause significant mortalities of the fly larvae, then it is assumed that there is no β-exotoxin present. Positive controls are included in the test containing the lowest concentration of that is expected to reliably kill 100% of the larvae. In this study the presence of β-exotoxin was investigated in 3 production batches of the technical slurry and two reference standards for β-exotoxin were used as positive controls. All determinations were conducted in triplicate.

The house fly bioassay is conducted in plastic cups. The house fly medium is composed of wheat bran and brewer yeast, 3:1, + 0.228% methyl paraben to control mould. Samples are prepared by autoclaving for 30 minutes, which kills any Bt spores and denatures any Bt endotoxins, but leaves the β-exotoxin unchanged. Liquid samples (1.5 mL) are mixed with 13.5 mL of water in the bioassay cup to a final volume of 15 mL. Dry samples need to be suitably diluted for the test. Positive controls are preparing a dilution of so that the concentration of β-exotoxin in the medium is 2 µg/mL. Place 12.5 g of the dry media into each cup containing the sample or standard and mix well. The fly eggs are added in a water suspension of 50 eggs/mL. While agitating the solution dispense 1 mL into each of the bioassay cups. Cover the cup with a plastic lid, which has been perforated to allow air exchange and incubate at 27-28 °C for 5 days. Count the number live larvae in each cup. If β-exotoxin is present the fly larvae will be dead, if there is no β-exotoxin present then there should be 25 to 50 live larvae.

Findings: The results of the testing are summarised in Table 4.3.5-01 and show that that the three slurry batches and the untreated control all contained living fly larvae. The two positive controls did not contain any live fly larvae. The result confirms that the three batches of XenTari fermentation slurry do not contain β-Exotoxins.

Table 4.3.5-01
Number of house fly larvae found after 5 days incubation

Sample Tested	Number of live larvae			
	Rep. 1	Rep.2	Rep.3	Mean
β-Exotoxin Type I	0	0	0	0.0
β-Exotoxin Type II	0	0	0	0.0
XenTari Fermentation Slurry run 2734	25	24	24	24.3
XenTari Fermentation Slurry run 2735	25	27	35	29.0
XenTari Fermentation Slurry run 2736	18	32	14	21.3
Untreated control	22	31	35	29.3

Conclusion:	**The study presents a method that is suitable for determining the presence of β-Exotoxin in XenTari fermentation slurry. No β-Exotoxin was detected in any of the three batches of XenTari fermentation slurry that were tested.**

Methods for the determination of enterotoxin by TECRA Bacillus Diarrhoeal Enterotoxin Visual Immunoassay (Bowman 2004) were developed and validated. The method was submitted and described under C.1.1.2 in Annex C: Confidential Information of the DAR (2007). A brief description of the method is provided below;

Report 4:	IIM 1.4.2/09. Bowman, L. (2004) (This report was previously considered in the DAR 2007.)
Title:	Summary report: Detection of enterotoxin in Valent BioSciences Bt fermentation beers and BT products.
Document No.	Valent Biosciences Corporation, Unpublished report No.: Not available
Guidelines:	Not stated
GLP:	No

Abstract:

The test method used was the TECRA *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay produced by Tecra International Pty Ltd. The TECRA BDE VIA system detects the 40-45 kDa protein referred to as the BDE Nonhemolytic enterotoxin or NHE. The results showed that both tests on the final fermentation beer and final XenTari product were negative.

Conclusion:

The TECRA VIA test is specific and sensitive method suitable for the detection of soluble enterotoxin protein in production beers and finished products of *Bacillus thuringiensis.*

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report:	L. Bowman (2004) Summary Report: Detection of enterotoxin in Valent BioSciences Bt fermentation Beers and BT products. Valent Biosciences Corporation, Unpublished report No.: Not available Unpublished report, not numbered
GLP:	No
Specificity:	Detection of soluble enterotoxin proteins in Bt fermentation beers and formulated product.

Method: The test method uses the TECRA Bacillus Diarrhoeal Enterotoxin Visual Immunoassay produced by Tecra International Pty Ltd. The TECRA BDE VIA system detects the 40-45 kDa protein referred to as the BDE non-hemolytic enterotoxin or NHE. This immunoassay test is extremely specific tests for the enterotoxin and is capable of detecting contamination >1 ng/mL in prepared samples.

Evaluation of Production Beers (EXP 336)

Final fermentation beers are store frozen and thawed just prior analysis. Samples are centrifuged for 20 minutes at 15000 rpm (26,900 g) and the supernatant filtered through a 0.2 µm filter. If centrifugation does not clarify the samples, they should be re-centrifuged for 40 minutes prior to the filtration step. The supernatant is diluted with 25 mMolar Tris buffer at pH 8.0 and analysed using the Tecra VIA test kit following the manufactures instructions.

Evaluation of Finish Products (EXP 377)

For liquid products, a sample is centrifuged and a 1/10 dilution is made from the supernatant and analysed using the Tecra VIA test kit following the manufactures instructions.

For technical powders and solid formulations, a solution in 25 mM Tris buffer is prepared, centrifuged and filtered through a 0.2 µm filter. Dilutions of 1/10 and 1/100 of the sample are made and analysed using the Tecra VIA test kit following the manufactures instructions.

Findings: **Evaluation of Production Beers (EXP 336)**

The method was evaluated using beer from production runs of 3 different strains of *Bacillus thuringiensis* and final fermentation beers from *Bacillus sphaericus* (strain 2362). In addition *Bacillus cereus* culture was run as a positive control. All of the Bt stains, including XenTari containing *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857 showed a negative response to the test. The positive control of *B. cereus* showed a strong positive reaction even at 1/100 dilution. The stability of the soluble enterotoxin protein was also investigated by analysing the *B. cereus* broth before and after freezing, which showed only a slight decline in response as a result of freezing the broth prior to analysis. The results are summarised in Table 4.3.5-02.

Evaluation of Finish Products (EXP 377)

The method was evaluated using finished products from 5 different strains of *Bacillus thuringiensis*. In addition *Bacillus cereus* culture was run as a positive control. All of the Bt stains, including XenTari containing *Bacillus thuringiensis* subsp. *aizawai*, strain ABTS-1857 showed a negative response to the test. The positive control of *B. cereus* showed a strong positive reaction. The results are summarised in Table 4.3.5-03

Table 4.3.5-02
Result of TECRA VIA enterotoxin test for final beers and broth of various *Bacillus*

Final beer or broth	Dilution			
	0	1/5	1/10	1/100

B. cereus (broth)	5	5	5	4
B. cereus (broth after freezing)	4	4	4	3
Bt aizawai (XenTari 2672) *	1	1	1	1

Test result of 3 to 5 is positive, 1 or 2 is negative
* production batches of final beer

Table 4.3.5-03
Result of TECRA VIA enterotoxin test for finished products of various *Bacillus*

Final beer or broth	Dilution		
	0	1/10	1/100
B. cereus (broth)	5	5	
Xentari DF (*Bt* subsp. *aizawai*, Strain ABTS-1857, Lot # 108-234-PG)	-	2	2

Test result of 3 to 5 is positive, 1 or 2 is negative

Conclusion: The TECRA VIA test is specific and sensitive method suitable for the detection of soluble enterotoxin protein in production beers and finished products of *Bacillus thuringiensis*.

Non-microbial impurities in the technical active substance are considered relevant if they are particularly undesirable because of their toxicological, ecotoxicological or environmental properties. None of the non-microbial impurities are considered relevant.

B.5.1.7 Methods to control the absence and to quantify (with appropriate limits of determination) the possible presence of any human and mammalian pathogen

In the addendum of the DAR (2012) the methods for the determination of relevant microbial impurities in the manufactured material are described

The methods to determine possible microbial impurities are reported as Confidential Information by Brand (2004) and Copeland (1990). The report allows the detection and quantification of a number of specific organisms, i.e.:

- *Staphylococcus aureus*
- *Clostridium perfringens*
- Coliform bacteria
- Salmonella
- *Pseudomonas aeruginosa*
- Standard aerobic microorganisms
- *E. coli*

- *St. aureus*.
- *Sal. enteriditis*
- *Enterococcus*
- Yeasts and moulds

The methods are specifically for the analysis of the WG formulation but they should also be suitable for analysing the technical powder or the slurry.

Possible microbiological impurities

Methods exist for the determination of the following impurities; Salmonella spp., Shigella spp., Staphylococcus aureus, Pseudomonas aeruginosa, Enterococci spp., E. coli Total Coliform, Listeria monocytogenes and Vibrio spp and were described in Volume 4, Annex C: Confidential Information, Section C.1.1.3. A brief description is provided below;

Report:	IIM 1.4.2/03. Brand, R.A. (2004) (This report was previously considered in the DAR 2007.)
Title:	Bioburden analysis of XenTari WG.
Document No.	Abbot Laboratories, Unpublished report No.: 78-134-04
Guidelines:	Not stated
GLP:	Yes

Abstract:

The report describes microbial methods for the detection and quantification of a number of specific organisms (*Salmonella spp*., *Shigella spp*., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococci spp*., *E. coli Total Coliform*, *Listeria monocytogenes* and *Vibrio spp.*). The methods reported are specifically for the analysis of the WG formulation but there is no scientific reason why they should not be suitable for analysing the technical powder. The methods include;

STM. 0154600	*Staphylococcus aureus*
STM. 0154700	*Enterococci*
STM. 0155400	*Clostridium perfringens*
STM. 0309600	Coliform bacteria
STM. 0323700	Salmonella
STM. 0323700 (B) Alternative method using 1-2 TEST®	Salmonella
STM. 0323800	*Pseudomonas aeruginosa*
STM. 0329100	Total aerobic microbial count
STM. 0545900	Yeast and mould count

Conclusion:

Well known microbiological methods exist for the determination of contaminating micro-organisms. Further validation is not required as they are standard pharmacopeia methods.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report:	R.A. Brand (2004) Bioburden analysis of XenTari WG. Abbott Laboratories, Unpublished report No.: 78-134-04
GLP:	Yes

This report contains microbial methods for the detection and quantification of a number of specific organisms. The methods are specifically for the analysis of the WG formulation but there is no scientific reason why they should not also be suitable for analysing the technical powder or the slurry. The methods are summarised below:

Method	STM. 0154600
Specificity:	*Staphylococcus aureus* in formulated product
Method:	The sample (10 mL or 1 g) is added to 90 mL of sterile USP phosphate buffer and mixed. Sub-sample of the suspension (1 mL) is transferred to sterile pre poured Baird-Parker agar plates supplemented with egg yolk tellurite enrichment. The inoculum is spread evenly across the surface of the plates and allowed to soak into the surface of the agar. The plates are inverted and incubated at 35-37°C for 21-26 hours. *Staphylococcus aureus* form characteristic black, shiny, convex colonies, surrounded by a clear zone in the Baird-Parker agar. If negative the plate should incubated for a further 24 hours and re-read.
Confirmation:	Gram stain test on typical colonies of should show Gram-positive cocci occurring in clusters. If colonies do not match this description then the test is negative. If it does meet the description than the coagulase test should be performed. Coagulase Test involves transferring a portion of the suspect colony to 0.5 mL of mammalian plasma under sterile conditions. Positive and negative controls should be included in the test. The tubes should be incubated at 35-37°C and examined at 3 hours and at suitable intervals up to 24 hours. If no coagulation of the plasma is observed in the suspect colony it does not contain coagulase positive *Staphylococcus aureus*. Additional biochemical or cultural tests may be performed if further confirmatory evidence is required.
Conclusion:	**Standard test method 0154600 provides a suitable method for the detection and quantification of *Staphylococcus aureus* in XenTari WG formulated product.**

Method	STM. 0154700
Specificity:	Enterococci in formulated product
Method:	Prepare Bacto m-enterococcus agar according to manufactures instructions. Do not autoclave. The sample (10 mL or 1 g) is added to 90 mL of sterile USP phosphate buffer, mixed and diluted as required to obtain 30 to 300 colonies per plate. Duplicate sub-samples of the suspension (1 mL) are transferred to sterile Petri dishes and promptly mixed with 15 to 20 mL of m-enterococcus agar, cooled to approximately 46-50°C. The dishes are covered and the contents mixed by tilting and rotating the dishes. The contents are allowed to solidify before inverting and incubat-

	ing the plates at 35-37°C for 24-48 hours. Typically enterococci produce pink to dark maroon colonies. The average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.
Confirmation:	Gram stain test on typical colonies of should show Gram-positive spherical or ovoid cocci occurring in chains. If colonies do not match this description than the test is negative. If it does meet the description than further confirmation can be obtained by employing the Vitek system or API 20S Streptococcus system or by performing some other acceptable biochemical test.
Conclusion:	**Standard test method 0154700 provides a suitable method for the detection and quantification of enterococci in XenTari WG formulated product.**

Method	STM. 0155400
Specificity:	*Clostridium perfringens* in formulated product
Method:	Prepare and sterilise Oxoid Perfringens agar, No. CM 543. Allow to cool to 50°C and add the Perfringens agar O.P.S.P. supplements A and B, which have been re-hydrated in 2 mL of sterile USP water. The agar should be mixed and held at 46-50°C until sample plates are ready. The sample (10 mL or 1 g) is added to 90 mL of sterile USP phosphate buffer, mixed and diluted as required to obtain 30 to 300 colonies per plate. Duplicate sub-samples of the suspension (1 mL) are transferred to sterile Petri dishes and promptly mixed with 15 to 20 mL of Perfringens agar, cooled to approximately 46-50°C. The dishes are covered and the contents mixed by tilting and rotating the dishes. The contents are allowed to solidify before incubating the plates anaerobically in an upright position at 35-37°C for 18-24 hours. *Clostridium perfringens* produce large black colonies (2-4 mm in diameter) within the agar. The average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.
Confirmation:	After confirming strict anaerobiosis the organism can be identified via the Vitek identification system using ANI cards.
Conclusion:	**Standard test method 0155400 provides a suitable method for the detection and quantification of *Clostridium perfringens* in XenTari WG formulated product.**

Method	STM. 0309600
Specificity:	Coliform bacteria in formulated product
Method:	The sample (10 mL or 1 g) is added to 90 mL of sterile USP phosphate buffer and mixed. Duplicate sub-samples of the suspension (1 mL) are transferred to sterile Petri dishes and promptly mixed with 15 to 20 mL of violet red bile agar, previously melted and cooled to approximately 46-50°C. The dishes are covered and the contents mixed by tilting and rotating the dishes and allowed to solidify at room temperature. The surface of the solidified agar is overlaid with an additional 3-4 mL of agar to

inhibit the formation of surface colonies. The plate are inverted and incubated at 35-37°C for 20-24 hours. Coliform colonies will appear dark red or purplish red in colour, about 1-2 mm in diameter and deep colonies appear lens shaped. The average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.

Confirmation: Gram stain test and oxidase test should be performed to confirm identity of coliform bacteria colonies. Coliforms will show Gram-negative rods and oxidase negative. Further identification can be obtained using the API 20 Enterobacteriaceae screening or by performing some other acceptable biochemical or cultural test.

Conclusion: **Standard test method 0309600 provides a suitable method for the detection and quantification of coliforms in XenTari WG formulated product.**

Method STM. 0323700 (A)

Specificity: Salmonella in formulated product

Method: Add 10 mL or 10 g of the sample to 90 mL of lactose broth and incubate at 35-37°C for 24 hours. Pipette 1mL sub-samples of the incubated culture into separate tubes, one containing 10 mL selenite cystine broth and the other 10 mL of fluid tetrathionate and incubate the tubes at 35-37°C for 18-24 hours. Using inoculation loop, streak portions from both incubated selenite cystine and terathionate tubes to surfaces of prepoured brilliant green agar or Xylose-lysine desoxycholate (XLD) agar and bismuth sulfite agar Petri dishes. Cover, invert the dishes and incubate at 35-37°C for 24 hours. The bismuth sulfite plate should be incubated for 24 hours before examining. On brilliant green agar Salmonella will form small transparent, colourless, pink or off white colonies, surrounded by pink or red zone. On the xylose-lysine-deoxycholate agar Salmonella will form red colonies with or without black centres. On the bismuth sulfite agar Salmonella will form black or dark green colonies. If none of the colonies match the above descriptions then the sample does not contain Salmonella.
The average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.

Method STM. 0323700 (B). Alternative method using 1-2 TEST®

Specificity: Salmonella in formulated product

Method: Add 10 mL or 10 g of the sample to 90 mL of lactose broth and incubate at 35-37°C for 24 hours. Pipette 1mL sub-sample of the incubated culture into a tube containing 10 mL of fluid tetrathionate without iodine and incubate at 35-37°C for 18-24 hours. Prepare and inoculate the 1-2 TEST® as directed. A positive detection of Salmonella from the 1-2 TEST® will need to be confirmed using the following methods.

Confirmation:	If colonies matching the above description are found a gram stain test should be performed. Salmonella will be visible as gram negative, rods. If colonies do not match this description then the test is negative. If it does meet the description than further identification should be obtained. A cultural test can be conducted by transferring some of the culture from the 1-2 TEST® or some of the suspected colonies to a but slant tube containing 10 mL of triple sugar iron Agar. The transferred material should be streaked well below the surface, and the tubes incubated at 35-37°C for 12 to 24 hours. Salmonella organism will ferment the glucose and produce acid and some species will produce gas and H_2S. If Salmonella is indicated the organism should be identified using the API 20 Enterobacteriaceae Identification System or other biochemical or cultural reactions.
Conclusion:	**Standard test method 0323700 provides a suitable method for the detection and quantification of Salmonella in XenTari WG formulated product.**

Method	STM. 0323800
Specificity:	*Pseudomonas aeruginosa* in formulated product
Method:	The sample (10 mL or 10 g) is added to 90 mL of sterile USP phosphate buffer and mix well. Transfer duplicate sub-sample of the suspension (1 mL) onto the surface of sterile pre poured Cetrimide Agar plates. The inoculum is spread evenly across the surface of the plates, which are then covered, and the inoculum allowed to soak into the surface of the agar. The plates are inverted and incubated at 35-37°C for 48-72 hours. *Pseudomonas aeruginosa* will produce characteristic bluish or green colonies. The average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.
Confirmation:	Gram stain test and oxidase test should be performed to confirm identity of *Pseudomonas aeruginosa* colonies. *Pseudomonas aeruginosa* will show gram negative rods and be oxidase positive. Further identification can be obtained using other acceptable biochemical or cultural test or by using the API 20 Enterobacteriaceae Screening System for the identification of oxidase positive, gram negative, non fermenting rods.
Conclusion:	**Standard test method 0323800 provides a suitable method for the detection and quantification of *Pseudomonas aeruginosa* in XenTari WG formulated product.**

Method	STM. 0329100
Specificity:	Total aerobic microbial count in formulated product and environment
Method:	**Pour plate procedure** The sample (10 mL or 10 g) is added to 90 mL of sterile USP phosphate buffer, mix well and diluted as required to yield 30 to 300 colonies per plate. Transfer duplicate sub-sample of the suspension (1 mL) into sterile Petri dishes and add 15-20 mL of soybean-casein digest agar medium,

	that has been melted and cooled to approximately 46-50°C. Cover the Petri dishes and mix the sample with the agar by tilting and rotating. Allow the contents to solidify at room temperature before inverting and incubating at 30 to 35°C for 48 to 72 hours. The plates should be examined for growth and the average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample.
	Surface Swabs
	The above method can also be adopted to determine the total aerobic microbial count in 10 mL surface swab samples. Suitable sterile suspending fluids should be used such as Letheen broth medium, containing 1% sodium metaphosphate and lactose broth medium. Swab samples should be diluted appropriately before plating out using the above method. Surface swab samples that have been suspended in less than 5 mL can be plated out by pouring the entire contents (including the swap head) into the sterile Petri dish. When the number of colonies on the plate exceed 300 then results for swab samples should be reported as greater than 300.
	Spread plate method
	Samples should be prepared and diluted as for the pour plate method. A sub-sample (1 mL) is then applied to the surface of a sterile pre-poured soybean-casein digest agar plate and the spread evenly across the surface of the agar. The plate is covered and the sample allowed to adsorb into the agar before incubating the plate, without inverting, at 30 to 35°C for 48 to 72 hours.
Conclusion:	**Standard test method 0329100 provides a suitable methods for the detection and quantification of total microbial counts in XenTari WG formulated product and for environmental monitoring.**
Method	STM. 0545900
Specificity:	Yeast and mould count in miscellaneous samples
Method:	The sample (10 mL or 10 g) is added to 90 mL of sterile USP phosphate buffer and mix well and diluted as required to yield 30 to 300 colonies per plate. Transfer duplicate sub-sample of the suspension (1 mL) into sterile Petri dishes and add 15-20 mL of Rose Bengal agar medium, that has been melted and cooled to approximately 46-50°C. Cover the Petri dishes and mix the sample with the agar by tilting and rotating. Allow the contents to solidify at room temperature before inverting and incubating at 46 to 50°C for 5 to 7 days.
	The plates should be examined for growth and the average number of colonies should be determined, multiplied by any dilution factor and expressed as number of colonies per unit volume of sample
Conclusion:	**Standard test method 0545900 provides a suitable method for the detection and quantification of total yeast and mould count in XenTari WG formulated product.**

Report:	A. Copeland (1990) *Bacillus thuringiensis* water dispersible granules (ABG-6314) bioburden testing. Unpublished, Study No. 40161-40165
GLP:	Yes
Method:	This report conducts bioburden analysis on the formulated product for various microorganisms. No method details are included although the following method references are included for each organism.

E. coli	SP #C-5-1
St. aureus	SP #S-9-1
P. aeruginosa	SP #P-11-1
C. perfringens	SP #C-6-1
Sal. Enteriditis	SP #S-8-1
Enterococcus	SP #E-10-1

Findings:	The methods were used to analyse 5 lots of ABG-6314. The only positive detections recorded were for Enterococci organism. Although the methods have only been used on formulated product they should equally suitable for use on technical material.
Conclusions:	Suitable methods are available for the detection of contaminating organisms in the formulated product.

Each batch of technical slurry is tested by the EPA mammalian mouse safety test (40CFR180.1011). This test was described in C.1.1.2 in Annex C: Confidential Information of the DAR (2007) and considered acceptable. No further information is required. A brief description of the method is provided below;

Report:	IIM 4.3.6/01. Jacobsen, S.D. (1993) (This report was previously considered in the DAR 2007.)
Title:	*Bacillus thuringiensis*: Acute toxicity in mice, subcutaneous dosing.
Document No.	Novo Nordisk, Unpublished SOP No.: TOX-SM-0001.01/01
Guidelines:	Not applicable
GLP:	No

Abstract:

The method is designed to be compliant with the method recommended in the Code of Federal Regulations USA, 40 CFR; 180.1011, July 1, 1987. The method is designed at detecting the presence of *Bacillus anthracis*.

In this test a mouse is given a subcutaneous injection of the production slurry containing a concentration of $>10^6$ spores and observed for 7 days. If the mouse shows any sign of infection or injury the batch will be rejected. Each test is performed on 5 SPF mice, 4-5 weeks old and 18 to 22 g in weight, a control group of 5 mice is also included.

The test is negative if none of the five animals show evidence of infection or injury. If any mouse reacts the test is regarded as positive and will be repeated with another 5 mice.

Conclusion:

The method follows the standard method for the testing of *Bacillus thuringiensis* for human or mammalian pathogenic effects.

This report has been considered previously in the DAR. Relevant extracts from the DAR are included below for reference:

Report:	S.D. Jacobsen (1993) *Bacillus thuringiensis*: Acute toxicity in mice, subcutaneous dosing . Novo Nordisk, Unpublished SOP No.: TOX-SM-0001.01/01
GLP:	No
Method:	The method is designed to be compliant with the method recommended in the Code of Federal Regulations USA, 40 CFR; 180.1011, July 1, 1987. The method is designed at detecting the presence of *Bacillus anthracis*. Each test is performed on 5 SPF mice, 4-5 weeks old and 18 to 22 g in weight, a control group of 5 mice is also included. The mice are injected with a single dose in the back of the neck. The dose volume of 0.1 mL will be a diluted suspension of the sample containing 1 million spores. Animals are observed for 3-5 hours after dosing and then once daily for a period of 7 days. All signs of ill health and any behavioural changes will be recorded. The injection site will be palpated daily in order to detect any swelling. Animals showing abnormal clinical signs during the study will be killed by inhalation of CO2 and subjected to macroscopic pathology. Animals found dead will also be subjected to macroscopic pathology. All remaining animals will be killed on day 7. The test is negative if none of the five animals show evidence of infection or injury. If any mouse reacts the test is regarded as positive and will be repeated with another 5 mice.
Findings:	No findings are presented.
Conclusions:	**The method follows the standard method for the testing of *Bacillus thuringiensis* for human or mammalian pathogenic effects.**

The following new intraperitoneal/subcutaneous single dose is available:

Data point addressed:	KMA 5.2.2.3/01
Author(s) (year):	Doig, A.J. (2015)
Title:	Microbial Pest Control Agent (MPCA) Safety Test in Mice *Bacillus thuringiensis* subsp. *aizawai*
Laboratory report / project number:	18643-15, 18673-15, 19188-15, 19495-15
Testing facility:	Stillmeadow, Inc.
Published:	No
Test guideline used:	OPPTS 885.1300
Deviations:	Not stated
GLP:	No

Objective: The objective of the study was to determine the safety of *Bacillus thuringiensis* subsp. *aizawai* in mice.

Methods: Five lots of *Bacillus thuringiensis* subsp. *aizawai* were tested, using five female mice per lot (ICR (CD-1); Texas Animal Specialities; 17-23 g). For each lot, 25.0 mg of the test substance was diluted to 50 mL with sterile saline. The site of injection was sterilised with 70% ETOH. The test substance/saline dilution was administered by subcutaneous injection in a single dose of 0.25 mL to each of 5 mice. Body weights were taken before dosing and at the end of the study to determine weight loss, if any. Initial observations were done in the cage or primary enclosure, noting any behavioural abnormalities, and checking for abnormal excrement or discharge. Physical examination of the animal was made beginning with the nose and observing the body of the animal to the tail. Any signs of toxicological/physiological abnormalities were noted and described. The injection site was observed for abnormal swelling, irritation or necrosis, and scored according to the Draize system if necessary.

Results: For all five lots of *Bacillus thuringiensis* subsp. *aizawai*, the results were determined to pass, concluding no unacceptable effects of the test substance on mice.

B.5.1.8 Methods to determine storage stability, shelf-life of the micro-organism, if appropriate

B.5.2 Methods to determine and quantify residues (viable or non-viable) of the active micro-organism

No specific MRL was fixed for the active substance under Reg. (EC) No 396/2005, according to Art. 18(1)(b) of that Regulation. Up till now Bacillus thuringiensis subsp. aizawai strain GC-91 is not included in Annex IV due to delay at EFSA. Moreover, the default MRL of 0.01 mg/kg is not applicable because agencies are not used to follow enforcement or maintenance procedures for micro-organisms. Furthermore, the evaluation of the renewal to still going on to disprove the EFSA opinion that Bacillus thuringiensis subsp. aizawai strain (ABTS 1857) and pathogenic B. cereus strain are comparable.

Provided that no MRLs are set in Reg (EC) No 396/2005, no analytical methods are required for monitoring of residues in food and feed as well as in water, soil and air samples."

Measurements of Cry proteins in the environment have gained more and more attention

B.5.2.1 The active micro-organism(s) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant (i.e. viable residues)

See B.5.2

B.5.2.2 Relevant metabolites (especially toxins) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant (i.e. non -viable residues)

See B.5.2

B.5.3 References relied on

See B.6 MA for summary literature search.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protec-tion claimed Y/N	Justification if data protection is claimed	Owner
KMA 4.1.6/06	Hernandez, C.S.	2001	Update on the detection of β-exotoxin in Bacillus thuringiensis strains by HPLC analysis Document No. Journal of Applied Microbiology 2001, 90, 643-647 GLP: No Published	N	N	-	Published