Ministry for Primary Industries
Manatū Ahu Matua

WPC2013 Response Report

Laboratory Identification of the Fonterra bacterial isolates

MPI Technical Paper No: 2013/28

Prepared for Ministry for Primary Industries
WPC2013 Response Management Team

ISBN No: 978-0-478-42027-2 (online)
ISSN No: 2253-3923 (online)

29 August 2013

Disclaimer

Considerable effort has been made to ensure that the information provided in this report is accurate, up to date, and otherwise adequate in all respects. Nevertheless, this information is made available STRICTLY on the basis that the New Zealand Ministry for Primary Industries, its employees, agents, and all other persons associated with the compilation, writing, editing, approval or publication of, or any other kind of work in connection with, the information:

1. disclaim any and all responsibility for any inaccuracy, error, omission, lateness or any other kind of inadequacy, deficiency, or flaw in, or in relation to, the information; and
2. without limiting (1) above, fully exclude any and all liability of any kind, on the part of any and all of them, to any person or entity (whether a user of this information or not) that chooses to rely upon the information.

While every effort has been made to ensure the information is accurate, the Ministry for Primary Industries does not accept any responsibility or liability for error of fact, omission, interpretation or opinion that may be present, nor for the consequences of any decisions based on this information. Any view or opinion expressed does not necessarily represent the view of the Ministry for Primary Industries.

The facts outlined in this report are those known at the time of finalising this report (29 August 2013) and are noted solely to identify the systems and processes undertaken by MPI in the confirmation of the identity of the bacterial isolates sourced from potentially contaminated product. MPI has initiated a compliance investigation, which is an entirely separate and independent process from this report.

Requests for further copies should be directed to:

Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
WELLINGTON 6140

Email: brand@mpi.govt.nz
Telephone: 0800 00 83 33
Facsimile: 04-894 0300

This publication is also available on the Ministry for Primary Industries website at http://www.mpi.govt.nz/news-resources/publications.aspx

Contents

		Page
1	**Executive Summary**	**1**
2	**Introduction**	**3**
3	**Methodology**	**3**
3.1	Bacterial isolates	3
3.2	Phenotypic and biochemical tests	4
3.3	16S rRNA sequencing	5
3.4	PCR testing for toxin genes	5
3.5	Next Generation Sequencing	6
3.6	ELISA testing for toxins	7
3.7	Mouse bioassay for toxigenic *C. botulinum*	8
4	**Results**	**10**
4.1	Phenotypic and biochemical tests	10
4.2	16S rRNA sequencing	10
4.3	PCR testing for toxin genes	13
4.4	Next Generation Sequencing (NGS) for toxin genes	13
4.5	ELISA for toxins	17
4.6	Mouse bioassay for toxigenic *C. botulinum*	17
5	**Conclusion**	**18**
6	**References**	**19**
7	**Appendix: WPC 2103 Laboratory Testing Plan 19/08/2013**	**20**
8	**Appendix: Diagnostic Tests and their Interpretation**	**23**
9	**Appendix: Cultures received by MPI from AgResearch**	**24**
10	**Appendix: Traceability of laboratory testing at AHL on bacterial isolates**	**27**
11	**Appendix Preliminary report and laboratory documents from AgResearch to Fonterra Co-operative Dairy Company Limited**	**28**
12	**Appendix Fonterra summary report 3 08082013 and timeline**	**29**
13	**Appendix NVSL, USDA Report**	**30**
14	**Appendix CDC Report**	**40**

1 Executive Summary

Following notification from Fonterra Co-operative Group (Fonterra) to MPI that bacterial contaminants identified by AgResearch as likely to be *Clostridium botulinum* had been detected by Fonterra in base powder containing contaminated whey protein concentrate (WPC80), the Ministry for Primary Industries (MPI) WPC2013 Response Management team determined the AgResearch findings needed to be validated. The response technical team was requested on Saturday 3 August 2013 to identify the bacterial contaminants isolated by Fonterra and verify their stated ability to produce toxin. Validation of initial laboratory results is a normal process for emergency responses.

Fonterra and the Government owned Crown Research Institute AgResearch (AgResearch) were contacted on Saturday 3 August 2013 to make arrangements for the MPI's Animal Health Laboratory (AHL) to receive the bacterial cultures. AgResearch subcultured the bacteria on Monday 5 August 2013 and MPI arranged shipment of the cultures from Palmerston North to AHL at Wallaceville on Tuesday 6 August 2013. The bacterial cultures did not come directly from WPC80 but an end product from Fonterra Waitoa of which WPC80 was an ingredient. The bacterial cultures that MPI subjected to confirmatory testing as described in this report were derived from the same cultures as those AgResearch tested by mouse bioassay and reported as *C. botulinum.*

The scientists at AHL prepared a laboratory testing plan for identification work to be undertaken both at the AHL and by other expert international laboratories, in particular the mouse bioassay.

- To identify the bacterial strain, biochemical and phenotypic testing, 16S rRNA sequencing and phylogenetic analysis were undertaken at the AHL. This testing differentiates between *C. botulinum* Groups I (toxin types A, B and F), II (toxin types B, E and F), III (toxin types C and D) and IV (toxin type G). *C. botulinum* Groups I and II are important to human health and Group III is important to animal health.

- To identify the presence of bacterial toxin genes and toxin proteins, PCR tests for toxin genes (A, B, C, D, E and F), commercial ELISA for toxin antigens (C and D) and next generation sequencing were performed at the AHL.

- To identify whether the bacterial isolate is toxigenic the mouse bioassay was conducted at two expert laboratories, the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA and the National Veterinary Services Laboratories (NVSL), United States Department of Agriculture, Ames, Iowa, USA. The mouse bioassay is the only universally-accepted 'gold standard' test for identification of toxigenic *Clostridium*.

The laboratory testing plan was discussed with Dr Eric Johnson, Professor of Bacteriology, University of Wisconsin-Madison, USA, during a teleconference with Fonterra and MPI on 12 August 2013. The plan was shared with representatives of Fonterra and AgResearch on 19 August 2013. Changes to the laboratory testing plan were not proffered by Professor Johnson, AgResearch or Fonterra.

All three bacterial isolates (1a6, 2a9 and 3a1) were presumptively identified using biochemical and phenotypic test results as belonging to either *C. sporogenes* or *C. botulinum*

Group I and not another *Clostridium* species. This identification was confirmed by 16S rRNA sequencing and phylogenetic analysis.

Toxin gene PCR tests were negative for *C. botulinum* toxin types A, B, C, D, E and F. Next generation sequencing analysis revealed the bacterial strain had the greatest homology to *C. sporogenes*. The nontoxin-nonhaemagglutinin (NTNH) gene was absent and none of the sequencing reads mapped to toxin genes A, B, C, D, E, F and G. The absence of the NTNH gene and the toxin genes support the negative toxin gene PCR result and provide support for the conclusion that the bacterial strains do not contain the toxin genes required to produce primary toxin types A, B, C, D, E, F and G.

The NVSL mouse bioassay final results were received by MPI on 27 August 2013. All cultures were negative for botulinum neurotoxin by mouse bioassay after seven days anaerobic broth incubation.

The CDC final mouse bioassay results were received by MPI on 28 August 2013. There was no evidence of the presence of a botulinum neurotoxin producing species of *Clostridium* in any of the cultures submitted to them.

The name *C. botulinum* is conserved for toxigenic strains, and *C. sporogenes* for nontoxigenic strains (Taxonomic opinion 69, Int J Sys Bacteriol, (1999), 49, 339).

Based on the results presented above MPI concludes that all three bacterial isolates provided to the AHL by Fonterra through AgResearch are not toxigenic and are identified as *Clostridium sporogenes*.

2 Introduction

Following notification from Fonterra to MPI that bacterial contaminants identified by AgResearch as *C. botulinum* had been detected by Fonterra in base powder containing contaminated whey protein concentrate (WPC80), the MPI WPC2013 Response Management team determined the AgResearch findings needed to be validated. Validation of initial laboratory results is a normal process for emergency responses.

The response technical team was requested on Saturday 3 August 2013 to obtain cultures of the identified contaminant from Fonterra, identify the bacterial isolate and verify its stated ability to produce toxin.

The following describes the procedures used by MPI to confirm the identity of the bacterial isolates and their ability to produce toxin, and documents the MPI conclusions.

3 Methodology

3.1 BACTERIAL ISOLATES

The bacterial cultures that MPI subjected to confirmatory testing as described in this report were derived from the same cultures as those AgResearch tested by mouse bioassay and reported as *C. botulinum.*

Nine bacterial cultures representing three matrix-assisted laser desorption/ionization time-of-flight mass spectrometer **(**MALDI-TOF) protein profiles were received from AgResearch at the MPI AHL at Wallaceville on 6 August 2013. The cultures were couriered under anaerobic atmospheric conditions and received within 5 hours.

The bacterial cultures were accessioned as W13/1233 and numbered 1-9 (Table 3.1.1). Plates labelled Clost. sporog 1a6, 2a9 and 3a1 were the bacterial cultures provided from Fonterra to AgResearch. Please refer to the table in Appendix 10 for traceability of isolates.

The AHL does not hold reference strains of *C. botulinum.* AgResearch kindly provided the following control strains of *C. botulinum* that had been obtained and held at the Meat Industry Research Institute of New Zealand (MIRINZ) facilities at Ruakura, Hamilton, prior to 1998. MIRINZ merged with AgResearch in 1999.

C. botulinum ATCC 25763^{T} (Type A)
C. botulinum 17B (CSIRO)
C. botulinum Type Ca
C. botulinum ATCC 27517(Type D)
C. botulinum (Type E strain Beluga)
C. botulinum ATCC 23387 (Type 202F)

Note: Type C did not grow on subculture, Type D aligned with Group 1 *C. botulinum* and Type F was PCR positive for Type E. The provenance of these reference strains was poor.

Table 3.1.1: Bacterial cultures received from AgResearch.

MPI Identification	Labels on plates		Media
	Identification	Date of subculture	
W13/1233 – 1	1a6 streaked from original Fonterra sample	05/08/2013	BA
W13/1233 – 2	2a9 streaked from original Fonterra sample	05/08/2013	BA
W13/1233 – 3	3a1 streaked from original Fonterra sample	05/08/2013	BA
W13/1233 – 4	1a6 from AgResearch subculture	05/08/2013	BA
W13/1233 – 5	2a9 from AgResearch subculture	05/08/2013	BA
W13/1233 – 6	3a1 from AgResearch subculture	05/08/2013	BA
W13/1233 – 7	Clost. sporog 1a6	06/07/2013	TSA
W13/1233 – 8	Clost. sporog 2a9	06/07/2013	TSA
W13/1233 – 9	Clost. sporog 3a1	06/07/2013	TSA

BA – Columbia blood agar
TSA – Tryptic soy agar

3.2 PHENOTYPIC AND BIOCHEMICAL TESTS

In readiness for shipment to the USA the next day, fresh subcultures were prepared on 6 August 2013. Subcultures were taken from W13/1233-1 – 3 and 7- 9 onto sheep blood agar (SBA) plates and cooked meat broth (CMB) using a colony sweep technique to ensure adequate bacterial growth. W13/1233-1-3 and 7-9 were selected as they were earlier generations. The SBA plates were incubated anaerobically at 30^{o}C and 37^{o}C and aerobically at 37^{o}C. The CMB were incubated aerobically at 37^{o}C.

On 7 August 2013 further subcultures were set-up from all plates received from AgResearch from single colonies or, where single colonies were not apparent, a very small amount of bacterial growth was taken from the final streak line onto SBA and incubated aerobically (1 plate) and anaerobically (2 plates) at 37^{o}C. The CMB were incubated aerobically at 37^{o}C.

Three cultures (W12/1233-2, 6 and 7) were selected from the subcultures performed on 7 August 2013 for identification. The following tests were conducted; Gram stains, oxidase, catalase, API 20A, indole spot test, nitrate, H_2S, Nagler plate, litmus milk, fermentation of glucose, arabinose, fructose, lactose, maltose, mannitol, mannose, salicin, sucrose and xylose.

Biochemical and phenotypic results were compared to the identification table for characteristics of *Clostridium* species of clinical significance (Manual of Clinical Microbiology, 9th Edition, Volume 1, Chapter 57, p 890-891).

3.3 16S RRNA SEQUENCING

3.3.1 Initial16S rRNA sequencing

DNA extraction from W13/1233-1, 2, 3 and 7 was conducted from the bacterial cultures supplied by AgResearch upon arrival on 6 August 2013. Representative bacterial growth was taken from each culture. DNA was subsequently extracted using the QIAGEN QIAamp DNA mini kit as per the manufacturer's protocol for Gram-positive bacteria.

The extracted DNA was subjected to 16S rRNA PCR. The 16S rRNA was amplified in all cultures. The amplified PCR products were purified using SureClean and submitted to EcoGene for gene sequencing.

3.3.2 Further 16S rRNA sequencing

On 9 August 2013 further DNA extraction was carried out from all nine W13/1233 bacterial cultures (AHL subculture 7/8/2013) using the QIAGEN QIAamp DNA mini kit with minor modifications.

In brief, a few well isolated colonies from a fresh subculture on SBA incubated anaerobically at 37°C from 7 August 2013 were transferred into 1 mL PBS (pH 7.4). Following 2 min centrifugation at 14,000 x g, the supernatant was discarded. The remaining bacterial pellet was subjected to heat-inactivation at 110°C for 10 min. Then 180 µL Lysozyme was added to the sample and incubated at 37°C for 1hr. After a cool-down on ice, 200 µL buffer AL and 20 µL Proteinase K were added to the sample and incubated at 56°C for 30 min and at 95 °C for 1hr. After a further cool-down on ice, DNA extraction was carried out as per the manufacturer's protocol for Gram-positive bacteria with the addition of 200 µL Ethanol.

The resulting DNA was subjected to 16S rRNA PCR. In addition, DNA from all nine cultures was subjected to PCR testing for *C. botulinum* toxin genes (refer section 3.4).

The 16S rRNA was amplified in all cultures. The amplified PCR products from representative cultures W13/1233 2, 6 and 7 (cultures subjected to bacterial identification) were purified using SureClean and submitted to EcoGene for gene sequencing.

3.4 PCR TESTING FOR TOXIN GENES

3.4.1 Initial PCR testing for *Clostridium botulinum* toxin genes

DNA extraction for initial PCR testing for toxin genes is described in the above section (3.3.2).

All nine cultures were subjected to PCR testing for *C. botulinum* toxin genes A, B, C, D, E, and F. Both conventional (Solomon and Lilly 2001) and real-time PCR (Satterfield et al. 2010) were carried out to detect *C. botulinum* toxin genes A, B, E, and F. Another

conventional PCR was also used for *C. botulinum* toxin genes C and D (Nakamura et al. 2010; Takeda et al. 2005). Positive controls for toxin genes A, B, E provided by AgResearch were also included in PCR. Controls for C, D, and F toxin genes were not available.

3.4.2 Further PCR testing for *Clostridium botulinum* toxin genes

Further DNA extraction from W13/1233 1, 2, 3, 4, 6, and 7 (cultures submitted to CDC and NVSL) was carried out on 16 August 2013 to achieve higher DNA yields. For this, Trypticase-Peptone-Glucose-Yeast Extract (TPGY) broth was inoculated with the bacterial culture on 15 August 2013. The bacterial culture provided by AgResearch was used for samples W13/1233-4, 6, and 7 and the bacterial culture prepared by AHL on SBA on 7 August 2013 was used for samples W13/1233-1, 2, and 3. TPGY broth was incubated overnight anaerobically at 37^{o}C. As W13/1233-7 failed to grow in TPGY from 15 August 2013, it was decided to use TPGY broth from 12 August 2013 for W13/1233-7. DNA extraction using 1 mL of TPGY broth inoculated with each sample was carried out using the QIAGEN DNeasy Tissue and Blood kit as per the manufacturer's protocol for Gram-positive bacteria.

All cultures were subjected to PCR testing for *Clostridium botulinum* toxin genes A, B, C, D, E, and F as described in Section 3.4.1.

3.5 NEXT GENERATION SEQUENCING

Genomic DNA from W13/1233-1 AgResearch 5 August 2013 and from W13/1233-7 (AHL TPGY broth 12 August 2013) for sequencing was obtained following the standard Gram-Positive protocols with a QIAamp DNA Mini kit (sample 1Q) or GeneAid Presto Mini gDNA Bacteria Kit (sample GA7i). DNA quality was assessed via gel electrophoresis, Qubit, and Nanodrop. Sequencing libraries were prepared and sequenced on a GS Junior machine (Roche) according to the manufacturer's protocols.

De novo assembly of sequence data was performed using the Roche "GS De Novo Assembler" software. Mapping assembly was performed using the Roche "GS Reference Mapper" software. These programs designed for assembly of 454 data are also known as "Newbler".

Initial assembly (de novo & mapping) was performed individually for the two sequencing runs (1Q & GA7i); the results (contig number and percentage of reads mapping to genomes/plasmids/phage) were comparable for both sets. The two sets of reads were then combined for final analysis.

Visualization and searching of assembled sequence data was performed using the programs Geneious version R.6.1.6 and Blast2Go v.2.6.6. Reference sequences for mapping were obtained from Genbank either directly or through Geneious connection to NCBI.

Mapping results were generated as follows. Reference sequences were downloaded directly from Genbank as .FASTA files, or downloaded from Genbank through Geneious and exported as .FASTA files. "GS Reference Mapper" (also known as Newbler) was used to map the run read files (.sff format) to the reference files, using default settings. Mapping quality/statistics were taken from the "consensusResults" section of the "454NewblerMetrics.txt" output file; percent reference coverage was taken from the "profile"

tab in the mapping software after run completion, and saved to the "*RefStatus.png|txt" files. Mapping was visualized by using Geneious to map the Newbler output to the annotated .gb file of the reference sequence. This last step was necessary to visually compare the contigs generated by the mapping assembly to annotated genes/genomes, because the FASTA file format used by Newbler for the reference sequence contains no annotation information. All further analysis used the complete contig set output (454AllContigs.fna).

"GS De Novo Assembler" (also known as Newbler) was then used to assemble the run read files (.sff format), using default settings aside from setting "extend low coverage overlaps" to true. Mapping quality/statistics were taken from "454NewblerMetrics.txt" output. All further analysis used the complete contig set output (454AllContigs.fna).

Assembly of "unmapped" reads was performed using Newbler and the program "SFF Tools" (Roche) as follows. After a mapping assembly as described above, a list of unmapped reads was generated from the mapping output file "454ReadStatus.txt" with the linux command "`grep Unmapped 454ReadStatus.txt >unmapped.txt`". An SFF file of the unmapped reads was then generated using the command "`sfffile –o unmapped.sff –i unmapped.txt sff/*`". De novo assembly was then performed as described above, using the new "unmapped.sff" containing all the unmapped reads. Blast2Go analysis of the contents/species hits for unmapped contigs was performed using the current web version of Blast2Go, using the algorithm "blastx" to determine the closest matching *protein* sequences. Blast2Go only works on contigs between 500-6,000 bp; thus the largest contigs were not analyzed.

Searching for specific genes (toxins, toxin clusters, *ntnH*) was performed in Geneious as follows. The query sequence was extracted from the reference genome/plasmid/phage sequences. This sequence was then used as a blastn query against the de novo assembly of the combined reads (90 contigs containing 98.5% of the reads and basepairs of the combined dataset), using the default Geneious blastn settings. The reliability of this technique for finding the *ntnH* gene in a large dataset was validated by performing the same query against whole genome/plasmid/phage sequences containing all 6 primary toxin types (A,B,C,D,E,F) and verifying that it found all *ntnH* genes in these reference sequences.

3.6 ELISA TESTING FOR TOXINS

The sole ELISA test available to AHL was the Bio-X – antigen detection ELISA kit for the detection of *Clostridium botulinum* types C and D neurotoxins. This ELISA detects C and D neurotoxin complexes but cannot distinguish between toxins C and D. It does not detect other toxin types.

Three subcultures were derived from the AHL subculture W13/1233-1-3, two from AgResearch Ltd subcultures 5 August 2013 W13/1233-4 and 6 and one from Fonterra subculture 6 July 2013 W13/1233-7. All were cultured and incubated anaerobically in CMB for five days (14 August 2013 to 19 August 2013). *C. sporogenes* (NCTC isolate 532) was also cultured.

In addition, *C. botulinum* A, B, D, E and F controls derived from AgResearch were also grown anaerobically in CMB for five days (14 August 2013 to 19 August 2013). All cultures were grown at 30ºC and another set at 37ºC.

On 19 August 2013, bacterial culture supernatants were tested in the Bio-X ELISA, following the manufacturer's instructions.

3.7 MOUSE BIOASSAY FOR TOXIGENIC *C. BOTULINUM*

MPI enlisted assistance for the mouse bioassay from the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, as they are internationally recognized experts in *C. botulinum* affecting human health, and the National Veterinary Services Laboratory (NVSL), Animal and Plants Inspection Service, United States Department of Agriculture, Ames, Iowa, as internationally recognized experts in *C. botulinum* affecting animal health.

Bacterial cultures were prepared from first subcultures or from primary plates provided to the AHL (Table 3.7.1). The six CMB and six swabs were inoculated to be at the same generation number.

For technical reasons, CMB and swabs were not prepared from W13/1233-5, 8 and 9. Nevertheless, the three Fonterra isolates, 1a6, 2a9 and 3a1, were represented in the cultures shipped to CDC and NVSL. Cultures were shipped to the CDC and NVSL on 8 and 9 August 2013, respectively, and arrived on the 14 and 15 August 2013 (US zone time), respectively.

The mouse bioassay reference methods procedures used by CDC and NVSL are not described in this report.

Table 3.7.1: List of samples sent to CDC and NVSL.

Accession number	CMB sent to CDC	Swabs sent to CDC	CMB sent to NVSL	Swabs sent to NVSL
W13/1233 - 1	Inoculated on 7/8 from AgR sub 5/8	Inoculated on 8/8 from AHL sub 7/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 9/8 from AHL sub 7/8
W13/1233 – 2	Inoculated on 7/8 from AgR sub 5/8	Inoculated on 8/8 from AHL sub 7/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 9/8 from AHL sub 7/8
W13/1233 – 3	Inoculated on 7/8 from AgR sub 5/8	Inoculated on 8/8 from AHL sub 7/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 9/8 from AHL sub 7/8
W13/1233 – 4	Inoculated on 7/8 from AgR sub 5/8	Inoculated on 8/8 from AHL sub 5/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 9/8 from AHL sub 5/8
W13/1233 – 6	Inoculated on 7/8 from AgR sub 5/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 8/8 from AgR sub 5/8	Inoculated on 9/8 from AHL sub 5/8
W13/1233 - 7	Inoculated on 7/8 from Fonterra sub 6/7	Inoculated on 8/8 from Fonterra sub 6/7	Inoculated on 8/8 from Fonterra sub 6/7	Inoculated on 9/8 from Fonterra sub 6/7

4 Results

4.1 PHENOTYPIC AND BIOCHEMICAL TESTS

All three bacterial strains were Gram positive bacilli with sub-terminal spores typical of *Clostridium* and were strictly anaerobic, growing at 30°C and 37°C. The colonies at 24 hours incubation were pin-point to small 1-2 mm, slightly shiny, irregular edge with some flat swarming and slightly β-haemolytic.

The API 20A results were presumptive for an identification of *C. sporogenes* or *C. botulinum.* The API 20A database does not differentiate between Groups of *C. botulinum*.

The bacterial strains were also negative for the following individual tests; oxidase, catalase, spot indole, nitrate, arabinose, fructose, lactose, mannitol, mannose, salicin, sucrose and xylose. Positive results were obtained for glucose, maltose, aesculin, gelatin and H_2S. Equivocal results were obtained from Nagler plate and litmus milk. The additional tests together with the API20A results were presumptive for *C. sporogenes* or *C. botulinum* Group I.

4.2 16S RRNA SEQUENCING

16S rRNA sequencing was carried out on the Fonterra isolates and the AgResearch control strains to help place the isolates within the phylogeny of *Clostridium* species. It was observed that the 16S rRNA sequences of the 3 isolates and the AgResearch control "D" were identical to each other, and together varied from the AgResearch control "A" sequence by only 4 base pairs (Figure 4.2.1).

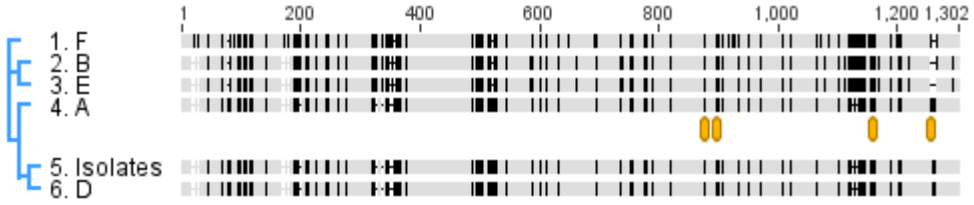


Figure 4.2.1: Alignment & Tree of the 16s rRNA sequences . Shown are the 3 Fonterra isolates & the AgResearch control strains. Black lines show where the 6 sequences differ. The 4 differences between "A" and the isolates/"D" are shown as yellow bars.

A simple 16S rRNA phylogenetic tree was used to place the isolates and control strains within the different Groups of *Clostridia* species (Figures 4.2.2 & 4.2.3). The Fonterra isolates clustered with Group I proteolytic *C. botulinum* / *C. sporogenes*. The AgResearch controls A, B, and E clustered as expected (Groups I, II, and II respectively). However, the AgResearch control D clustered with Group I, and control F was on its own with a closest match to Group II (Figure 4.2.3).

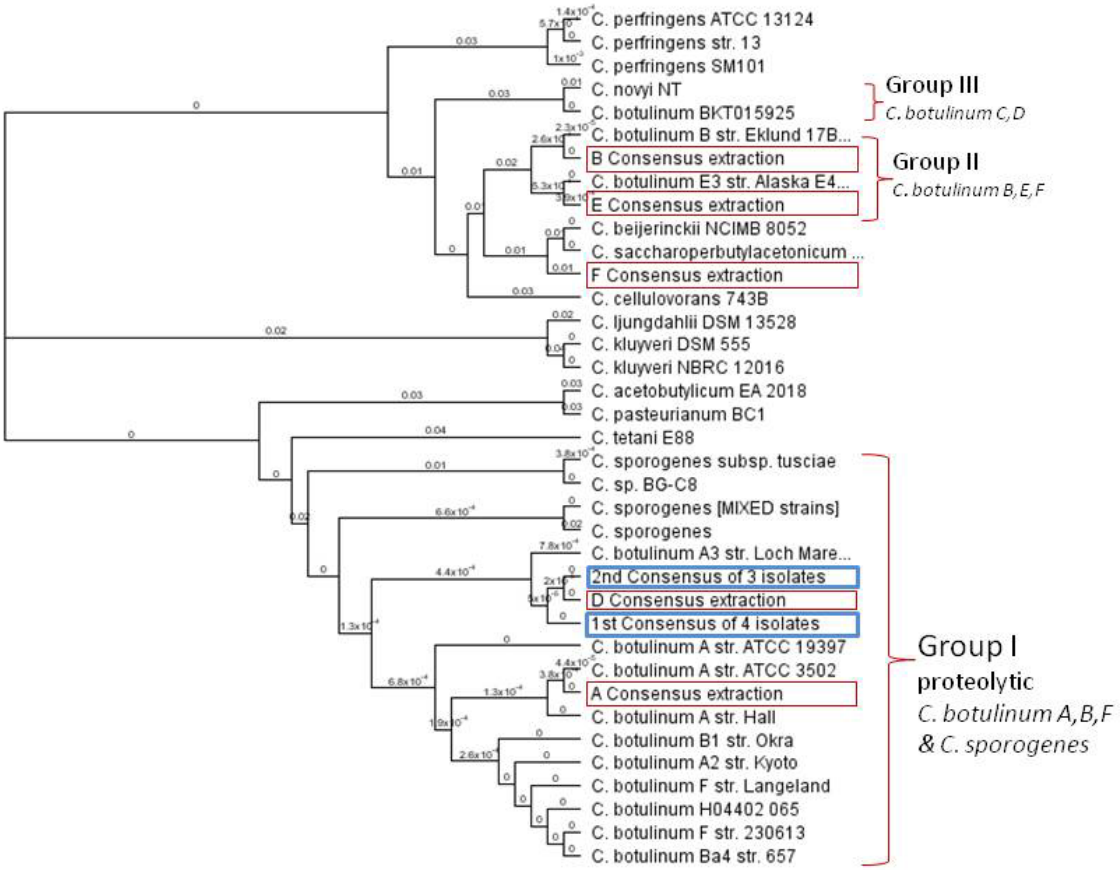


Figure 4.2.2: 16S rRNA phylogenetic tree. Tree generated in Geneious 5.5.6. The sequences from the Fonterra isolates are highlighted with blue bars and the AgResearch control strains are highlighted with red bars. Numbers on branches are “substitutions per site”.

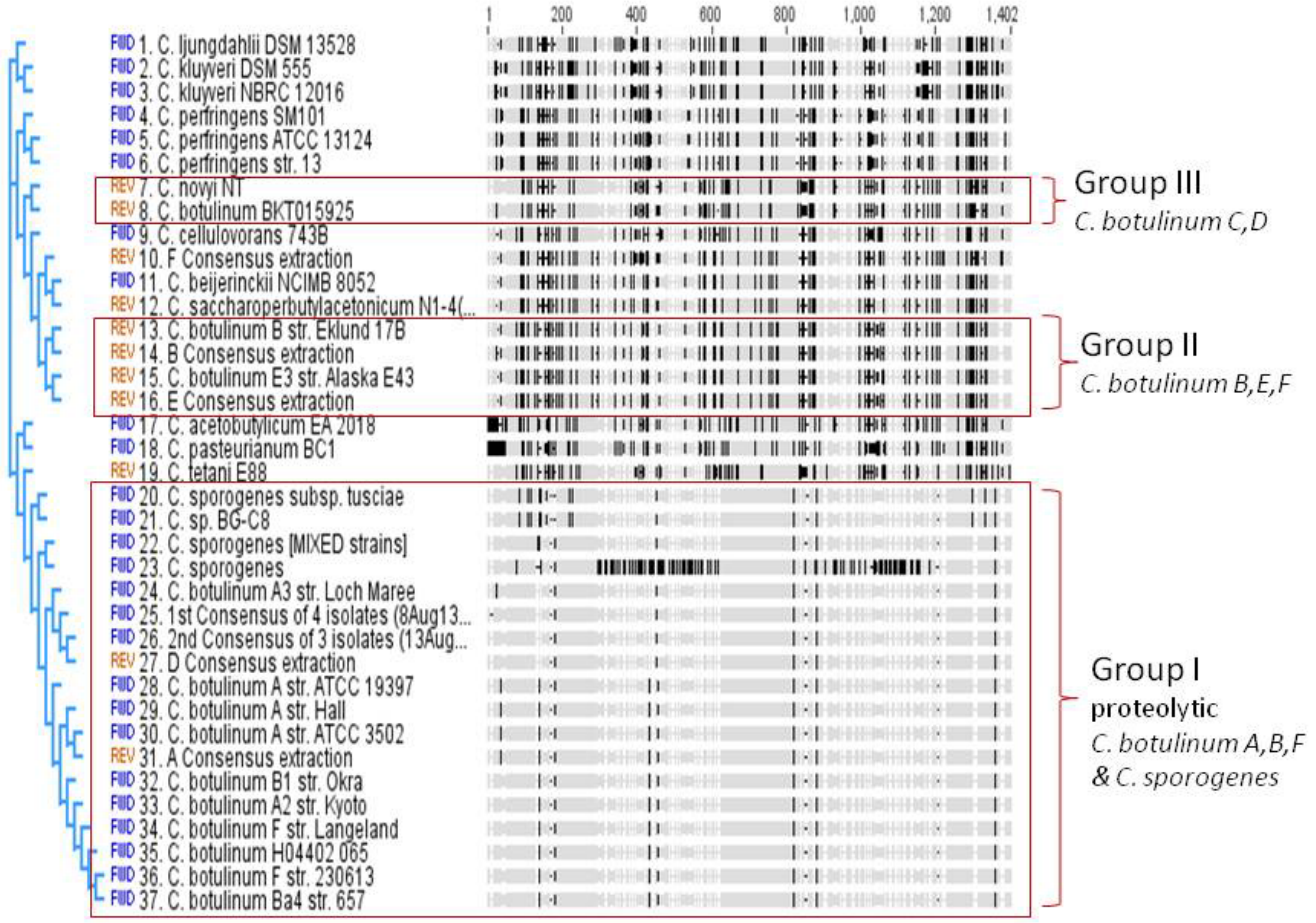


Figure 4.2.3: 16S rRNA phylogenetic tree – alignment view. Alignment view of the tree shown in Figure 4.2.2 above, demonstrating the sequence variation among the isolates. Black lines represent areas of sequence divergence.

4.3 PCR TESTING FOR TOXIN GENES

Genes for production of botulinum toxin types A, B, E and F were not detected in any of the cultures by conventional and real-time PCR (Table 4.3.1). In addition, genes for production of botulinum toxin types C or D gene were not amplified by conventional PCR (Table 4.3.1). Repeat testing was conducted on the cultures provided to CDC and NVSL using an improved procedure.

Table 4.3.1: Summary of DNA concentration and PCR testing for *C. botulinum* toxin genes.

	PCR testing for *C. botulinum* toxin genes (1st)							PCR testing for *C. botulinum* toxin genes (2nd)						
	DNA (ng/ µL)	A	B	C	D	E	F	DNA (ng/ µL)	A	B	C	D	E	F
W13/1233-1	0.38	N	N	N	N	N	N	27.8	N	N	N	N	N	N
W13/1233-2	0.37	N	N	N	N	N	N	27.6	N	N	N	N	N	N
W13/1233-3	0.44	N	N	N	N	N	N	7.2	N	N	N	N	N	N
W13/1233-4	0.43	N	N	N	N	N	N	43.6	N	N	N	N	N	N
W13/1233-5	0.39	N	N	N	N	N	N							
W13/1233-6	0.49	N	N	N	N	N	N	23.8	N	N	N	N	N	N
W13/1233-7	0.40	N	N	N	N	N	N	2.1	N	N	N	N	N	N
W13/1233-8	0.44	N	N	N	N	N	N							
W13/1233-9	0.42	N	N	N	N	N	N							

*N: negative

4.4 NEXT GENERATION SEQUENCING (NGS) FOR TOXIN GENES

The two 454 (GS Junior) runs produced data within the acceptable quality range for GS Junior, with the "1Q" run producing slightly higher-quality data (Table 4.4.1). As *C. botulinum* and related genomes tend to be in the range of 3-4 megabases, the combined datset represents ~ 36x coverage.

Table 4.4.1: NGS run results

Run	Useable Reads	Useable bp	Length Average (bp)
GA7i – 16-17/8/2013	158,778	62,974,590	397
1Q: 19-20/8/2013	170,369	81,416,001	478
Combined	329,147	144,390,591	438

De novo assembly statistics are shown in Table 4.4.2. All 90 contigs were used for analysis of the de novo assembly data.

Table 4.4.2: De Novo assembly statistics

	number	avg size	N50	largest	total bp assembled
Large Contigs (>500 bp)	60	68,624	148,548	384,907	4,117,495
All Contigs	90				4,124,624

Mapping was used to assemble the contigs to an assortment of reference genomes, as a way to determine the "closest match" of the sequence data to available genome sequence. The results of these mapping assemblies are summarized in Figures 4.4.1 & 4.4.2.

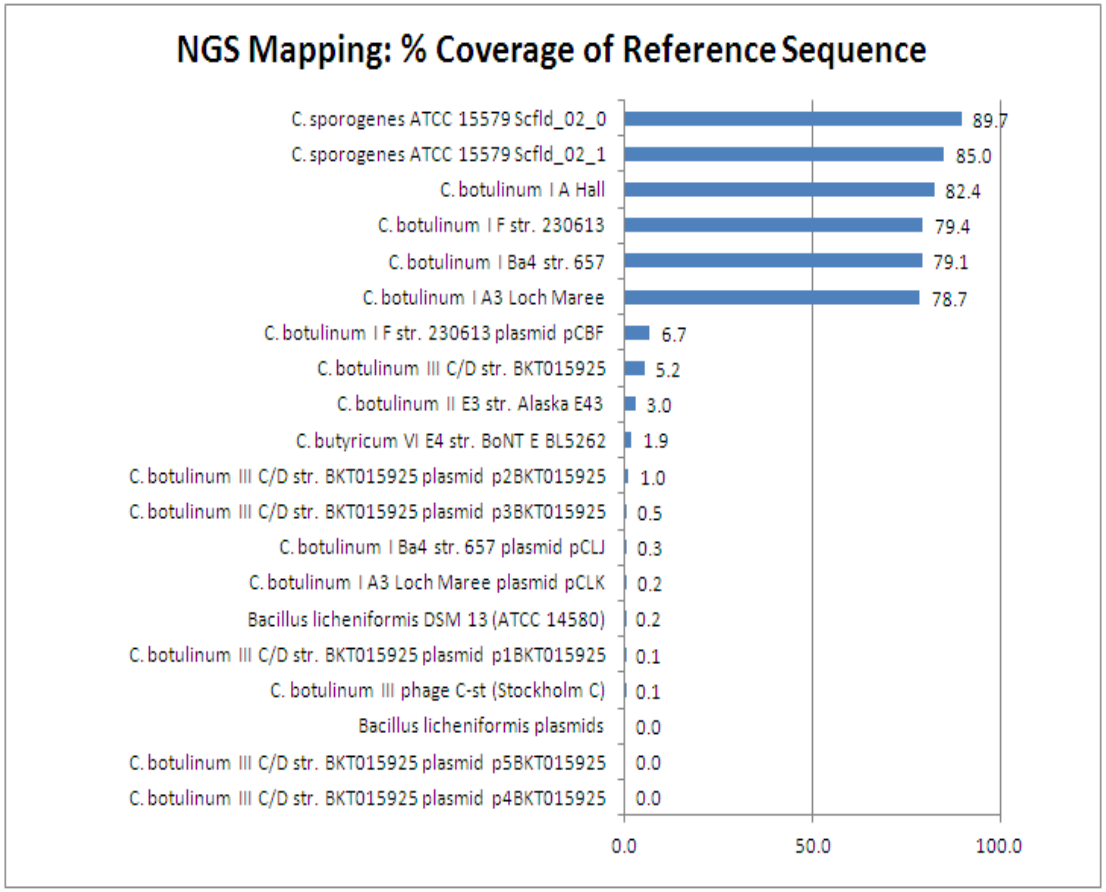


Figure 4.4.1: NGS mapping statistics

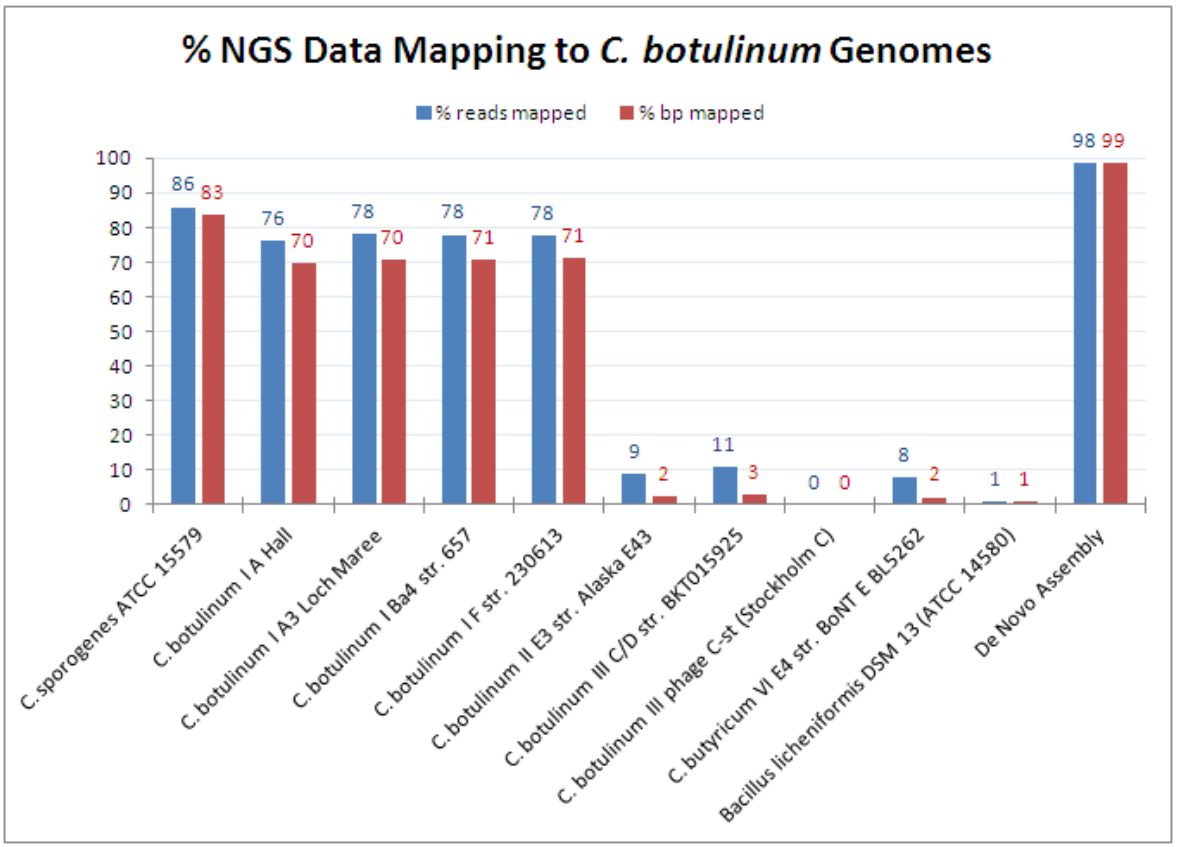


Figure 4.4.2: NGS mapping statistics

As a further approach to analyze the relationship of the reads to *C. botulinum*, a dataset containing all the reads that were "unmapped" to *C. sporogenes* ATCC 15579 and *C. botulinum* A str. Hall were extracted and assembled separately. The resulting assemblies were then submitted to Blast2Go to determine their closest matching organism against the Genbank database "nr". The results for contigs of usable size are shown in Figure 4.4.3.

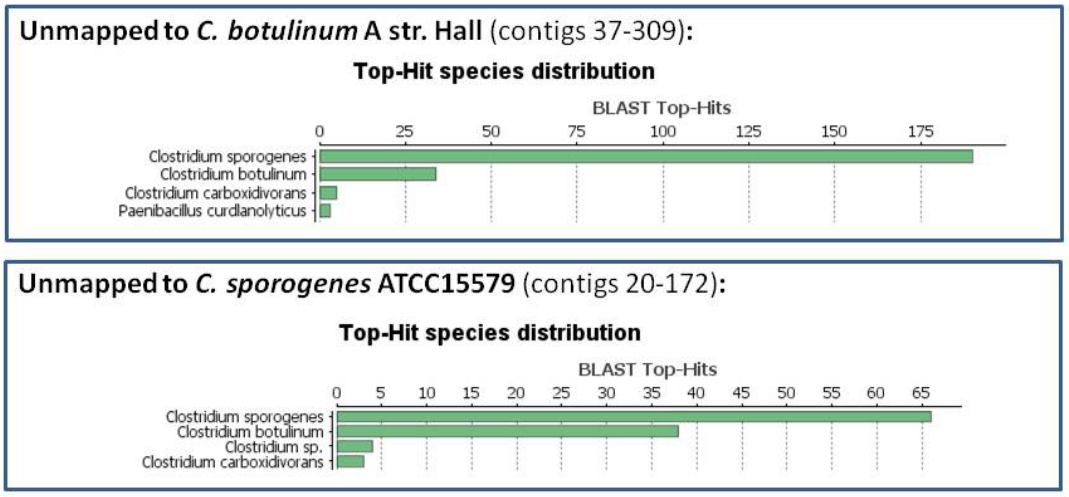


Figure 4.4.3: Best species matches to "unmapped" NGS data

Because the mapping assemblies failed to show reads mapping to the toxin gene clusters (see example in Figure 4.4.4), further work was undertaken to look for the presence of botulism toxin (BoNT) gene clusters in the sequence data.

Figure 4.4.4: Mapping Assembly *to C. botulinum* A str. Hall. Regions where NGS sequence maps to the reference genome (Accession CP00727) are shown in the ochre "Cover" graph. Top: coverage of the complete genome (82%). Bottom: closeup showing the lack of coverage over the ha toxin cluster (grey bar). Genes are shown as yellow arrows, and mapping contigs as black bars at the very bottom of the figure; white spaces in the black bars show regions of difference between the NGS sequence and the reference genome. A similar result is obtained for genomes containing orfX toxin clusters.

Botulism toxin (BoNT) gene clusters from all 7 toxin types (ABCDEFG) and all 6 *C. botulinum* "Groups" (I-VI, including *C. novii, C. baratii,* and *C. butyricum*), encode the gene NTNH, or "non-toxin non-hemagglutinin", inbetween the toxin and hemagglutinin/regulatory genes. This gene was thus used to search the de novo assemblies for the presence of potential botulism toxin gene clusters. The NTNH gene from *C. botulinum* A3 str. Loch Maree was used as a **blastn** query against the 90-contig de novo assembly, as well as a series of "positive controls" (genomes containing toxin clusters).

No significant matches to the NTNH gene were found in the de novo assembly or the *C. sporogenes* ATCC 15579 genome, whereas the search found the NTNH gene in genomes containing toxin clusters (Table 4.4.3).

Table 4.4.3: Sequences tested for the NTNH gene

NTNH (A3 Loch Maree) blastn *vs:*	Result	Found in:
De Novo Assembly (1Q & GA7i combined)	NEG	N/A
C sporogenes ATCC 15579	NEG	N/A
C botulinum I F str. 230613	POS	genome
C botulinum II B Elklund 17B	POS	plasmid pCLL
C botulinum II E Alaska E43	POS	genome
C botulinum III C/D BKT015925	POS	plasmid p1BKT015925
C botulinum I Group I plasmids	POS	pCLK (A3 Loch Maree); pCLJ (Ba4 str. 657); pCLD (B1 str. Okra)

To search for the toxin genes, the botulinum toxin (BoNT) gene clusters from all 7 toxin types (ABCDEFG) and all 6 *C. botulinum* "Groups" (I-VI, including *C. baratii* and *C. butyricum*) were used as **blastn** queries against the 90-contig de novo assembly; toxin clusters tested are listed in Table 4.4.4. Significant matches were not found to any of these clusters.

Table 4.4.4: Toxin clusters searched for in de novo assembly

BoNT Toxin Clusters Tested	Genbank Accession
A toxin cluster (ha) (Grp I) – *C. botulinum* str. A Hall	CP000727
A2 toxin cluster (orfX) (Grp I)- *C. botulinum* str. A2 Kyoto	CP001581
A3 toxin cluster (orfX) (Grp I)- *C. botulinum* str. A3 Loch Maree plasmid pCLK	CP000963
A4 toxin cluster (ha) (Grp I) - *C. botulinum* str. H04402 065	FR773526
B toxin cluster (ha) (Grp II) - *C. botulinum* str. Eklund 17B plasmid pCLL	CP001057
B1 toxin cluster (ha) (Grp I) - *C. botulinum* str. B1 Okra plasmid pCLD	CP000940
C toxin cluster (ha) (Grp III) - str. Stolkholm phage C-st	AP008983
C1 toxin cluster (ha) (Grp III) - *C. botulinum* C/D plasmid p1BKT015925	CP002411
D toxin cluster (ha) (Grp III) - *C. botulinum* D phage AB012112	AB012112
E3 toxin cluster (orfX) (Grp II) - *C. botulinum* str. E3 Alaska E43	CP001078
E4 toxin cluster (orfX) (Grp VI) – *C. butyricum* E4 str. BoNT E BL5262	NZ_ACOM00000000
F toxin cluster (orfX) (Grp I) - *C. botulinum* str. F Langeland	CP000728
F toxin gene (Grp V) - *C. baratii*	HM746656
G toxin gene (Grp IV)	X74162

In summary, comparisons of the genomic sequence of isolate 1a6 to completed *C. botulinum*-related genomes show a highest similarity to *C. sporogenes* ATCC 15579, followed by *C. botulinum* Group I strains. Botulism toxin genes were not found in genome data.

4.5 ELISA FOR TOXINS

All bacterial culture supernatants were negative for *C. botulinum* types C and D neurotoxins, including the *C. botulinum* toxin D control culture. This culture was suspected not to be a toxin D type (see PCR testing results). The positive control supplied with the ELISA kit gave a strong test signal.

These results showed that none of the bacterial cultures were of the toxin type C or D.

4.6 MOUSE BIOASSAY FOR TOXIGENIC *C. BOTULINUM*

The NVSL reported final results for the mouse bioassay on 27 August 2013. NVSL stated that all cultures were negative for botulinum neurotoxin by mouse bioassay after seven days of anaerobic broth incubation. Pure growth of colonies consistent with *Clostridium* was obtained from plate culture from all samples at the time of broth incubation, indicating all cultures were viable.

The CDC reported final results for the mouse bioassay on 28 August 2013. CDC stated that there was no evidence of the presence of a botulinum neurotoxin producing species of *Clostridium* in any of the cultures submitted to them.

5 Conclusion

The bacterial cultures isolated by Fonterra from base powder containing contaminated whey protein concentrate (WPC80) and provided to the AHL by AgResearch were identified as *Clostridium sporogenes*.

All three bacterial isolates (1a6, 2a9 and 3a1) were presumptively identified using biochemical and phenotypic test results as belonging to either *C. sporogenes* or *C. botulinum* Group I and not another *Clostridium* species. This identification was confirmed by 16S rRNA sequencing and phylogenetic analysis.

Toxin gene PCR tests were negative for *C. botulinum* toxin types A, B, C, D, E and F. Next generation sequencing analysis revealed the bacterial strain had the greatest homology to *C. sporogenes*. The nontoxin-nonhaemagglutinin (NTNH) gene was absent and none of the reads mapped to toxin genes A, B, C, D, E, F and G. The absence of the NTNH gene and the toxin genes support the negative toxin gene PCR and provide support for the conclusion that the bacterial strains do not contain the toxin genes required to produce primary toxin types A, B, C, D, E, F and G.

The NVSL mouse bioassay final results were negative for botulinum neurotoxin by mouse bioassay after seven days anaerobic broth incubation.

There was no evidence of the presence of a botulinum neurotoxin producing species of *Clostridium* in any of the cultures tested by mouse bioassay by CDC.

6 References

Nakamura et al. 2010. Characterization of the D/C mosaic neurotoxin produced by Clostridium botulinum associated with bovine botulism in Japan. Veterinary Microbiology 140: 147-154.

Solomon HM, Lilly T 2001. Chapter 17. Clostridium botulinum. In: Food and Drug Adminstration ed. Bacteriological Analytical Manual (BAM),. Online ed. Silver Spring, MD 20993.
http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm, Food and Drug Administration. Pp. 17.01-17.10.

Satterfield et al. 2010. A quadruplex real-time PCR assay for rapid detection and differentiation of the Clostridium botulinum toxin genes A, B, E and F

Takeda et al. 2005. Characterization of the neurotoxin produced by isolates associated with avian botulism. Avian Diseases 49:376-381.

7 Appendix: WPC 2103 Laboratory Testing Plan 19/08/2013

7.1 OBJECTIVES

To validate:

(i) the identification of the bacterial isolates,
(ii) the presence of toxin,
(iii) if the isolate(s) are toxigenic *Clostridium botulinum,* the type of toxin present.

7.2 INTERPRETATION CRITERIA

7.2.1 Presumptive for *Clostridium botulinum*

Clostridium botulinum is a Gram-positive, rod-shaped bacterium and endospores are oval and sub-terminal. They grow optimally at 30-37^{o}C in anaerobic atmospheric conditions, are motile and produce a small zone of β-haemolysis on blood agar. Positive results for glucose, H_2S production, lipase, gelatin and litmus milk. Negative results for lactose, salicin, xylose, nitrate, indole, meat (digestion), urea and lecithinase.

NOTE: Classical bacteriology testing is unable to differentiate between *C. botulinum* and *C. sporogenes*.

16SrRNA gene DNA sequences matches *C. botulinum* and *C. sporogenes*

NOTE: There appear to be phylogenetic differences between Clostridium Groups II-IV and *C. sporogenes*.

PCR assays detect toxin genes.

Whole genome sequencing detects toxin genes.

NOTE: None of the DNA-based methods are capable of indicating whether an isolate will produce botulinum toxin. Some methods, such as PCR identification of the presence of a botulinum neurotoxin gene, may indicate that the isolate **MAY** have the **capacity** to produce botulinum toxin but they are not proof that the isolate can actually produce the toxin[1].

ELISA positive for toxin.

NOTE: ELISA- based methods which are solely dependent on antigen detection and are not capable of identifying any enzymatic activity of botulinum toxin can only provide a suspected identification of the presence of botulinum neurotoxin[1].

7.2.2 Positive for *Clostridium botulinum*

Mouse bioassay (part 3) detects biologically-active botulinum neurotoxins.

NOTE: Positive results from a combination of methods may increase the likelihood of a botulinum toxin producing isolate even in the absence of a mouse bioassay result, for example—a PCR result (e.g. Type A gene detected) which completely matches an ELISA result (Type A toxin detected)[1].

7.2.3 Negative for *Clostridium botulinum*

Characteristics do NOT meet the criteria for positive toxin confirmatory test interpretation and/or another bacterial species is identified.

7.3 IDENTIFICATION OF THE ISOLATE

7.3.1 Bacteriology: involving identification, classification and characterisation based on phenotype and biochemical testing.

Estimated timeframe: 2 weeks

7.3.2 16SrRNA sequencing: involving PCR amplification and sequencing of the 16SrRNA gene and comparison to publicly available database of DNA sequences.

Estimated timeframe: 1 week

7.3.3 Genome sequencing: involving sequencing of the whole genome of the isolate and comparison to publicly available database of DNA sequences.

Estimated timeframe: 4 weeks

7.3.4 Presence of a toxin

Mouse bioassay (part 1): detects biologically active toxin. The assay requires a three part approach, the first part to screen for a toxin.

Estimated timeframe: 2 weeks

7.3.5 *C. botulinum* toxin typing

PCR: Range of tests to identify *C. botulinum* toxin type.

Estimated timeframe: 2 weeks

Mouse bioassay (parts 2 & 3): Following determination of the presence of a toxin, the toxin titre is determined (part 2) and the toxin identified by neutralisation using monovalent antitoxins (part 3).

Estimated timeframe: 3 weeks

ELISA for toxin types A, B, E and F: detects and differentiates toxin types A, B, E & F using polyclonal antibodies.

Estimated timeframe: 4 weeks

ELISA for toxin types C & D: one commercial kit that detects toxin types C & D.

Estimated timeframe: 4 weeks

Timeframes are approximate only and are contingent on shipping, bacteria viability and no requirement for repeat testing.

1. Advice provided by Dr XXXXXXXX, XXXXXXXXX, National Botulism Laboratory Team, Centers for Disease Control and Prevention.

8 Appendix: Diagnostic Tests and their Interpretation

Test	Result	Interpretation	Predictor of toxicity	Laboratory	Result date
Biochemical and phenoypic	*C. sporogenes*/*C. botulinum* Group 1.	The bacterium is either *C. sporogenes* or *C. botulinum* Group I and is not another Clostridial species. This testing does not differentiate between *C. sporogenes* and *C. botulinum* Group I.	No	MPI AHL Wallaceville	14/08/2013
16S rRNA sequencing	*C. sporogenes*/*C. botulinum* Group 1.	The bacterium is either *C. sporogenes* or *C. botulinum* Group I and is not another Clostridial species. This testing does not differentiate between *C. sporogenes* and *C. botulinum* Group I.	No	MPI AHL Wallaceville	14/08/2013
Toxin real-time and conventional PCR tests	PCR negative for Group 1 toxin types A, B and F. Also negative for toxin types C, D and E.	Confirmed that toxin genes are not present for Group I toxin types A and B. Provisional that toxin gene for F is not present as no PCR positive control was available.	Presumptive	MPI AHL Wallaceville	16/08/2013
Toxin ELISA	ELISA negative for toxin types C and D.	Confirmed that the bacterium does not produce toxin antigen for type C and D. Commercial ELISA not available for toxin types A, B, E and F.	Presumptive	MPI AHL Wallaceville	20/08/2013
Next Generation Sequencing	Greatest homology to *C. sporogenes*. Nontoxin-nonhaemagglutinin gene not present. No reads mapping to primary toxin genes A, B, C, D, E, F and G.	Absence of the NTNH and toxin genes supports the PCR test results that the bacterium does not have the toxin genes present to produce the primary toxin types A, B, C, D, E F and G. This together with the genetic homology is presumptive for *C. sporogenes*.	Presumptive	MPI AHL Wallaceville	21/08/2013
Mouse bioassay	Preliminary negative.	No evidence of the presence of a botulinum toxin-producing Clostridia species.	Presumptive	CDC NVSL	23/08/2013
Mouse bioassay	Confirmed negative	No evidence of the presence of a botulinum neurotoxin producing species of Clostridium in any of the cultures submitted to CDC and NVSL.	Confirmed	CDC NVSL	28/08/2013 27/08/2103

9 Appendix: Cultures received by MPI from AgResearch

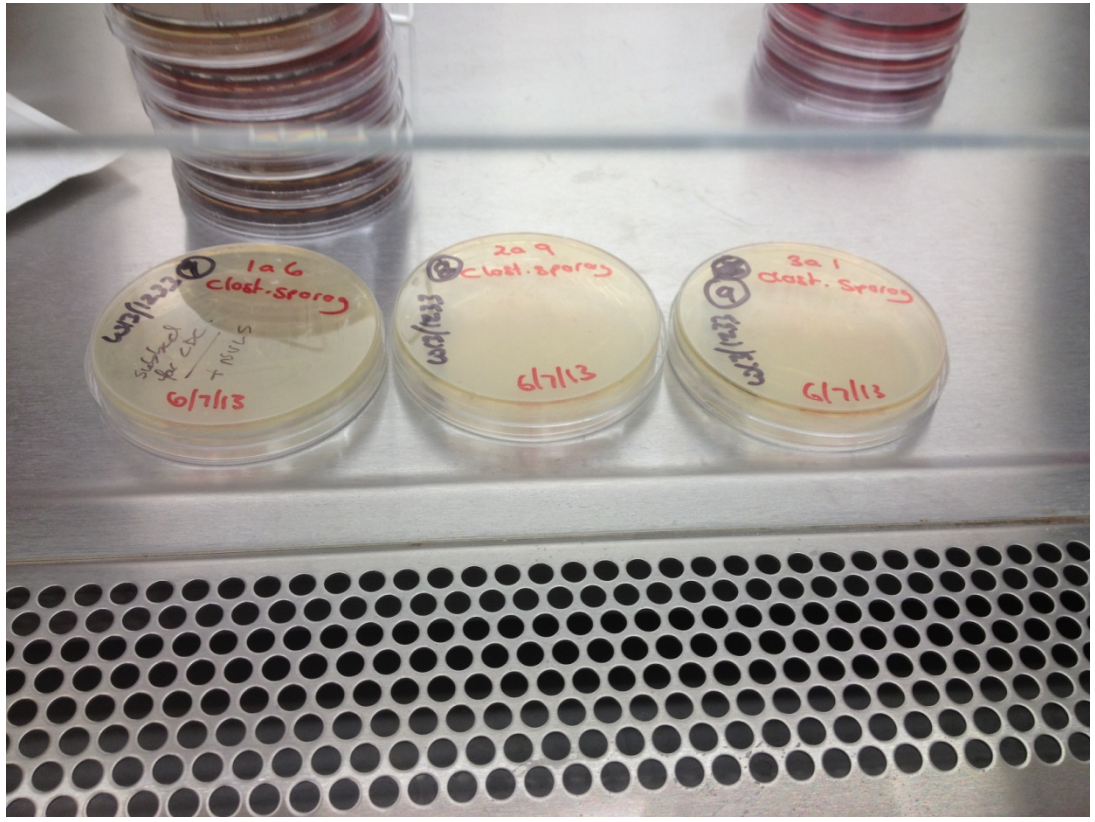


Figure 9.1: TSA plates provided to AgResearch from Fonterra and labelled 1a6, 2a9 and 3a1 Clost. sporog and dated 6/7/2013. AHL accession numbers W13/1233 7 – 9. Photograph taken on 27 August 2013.

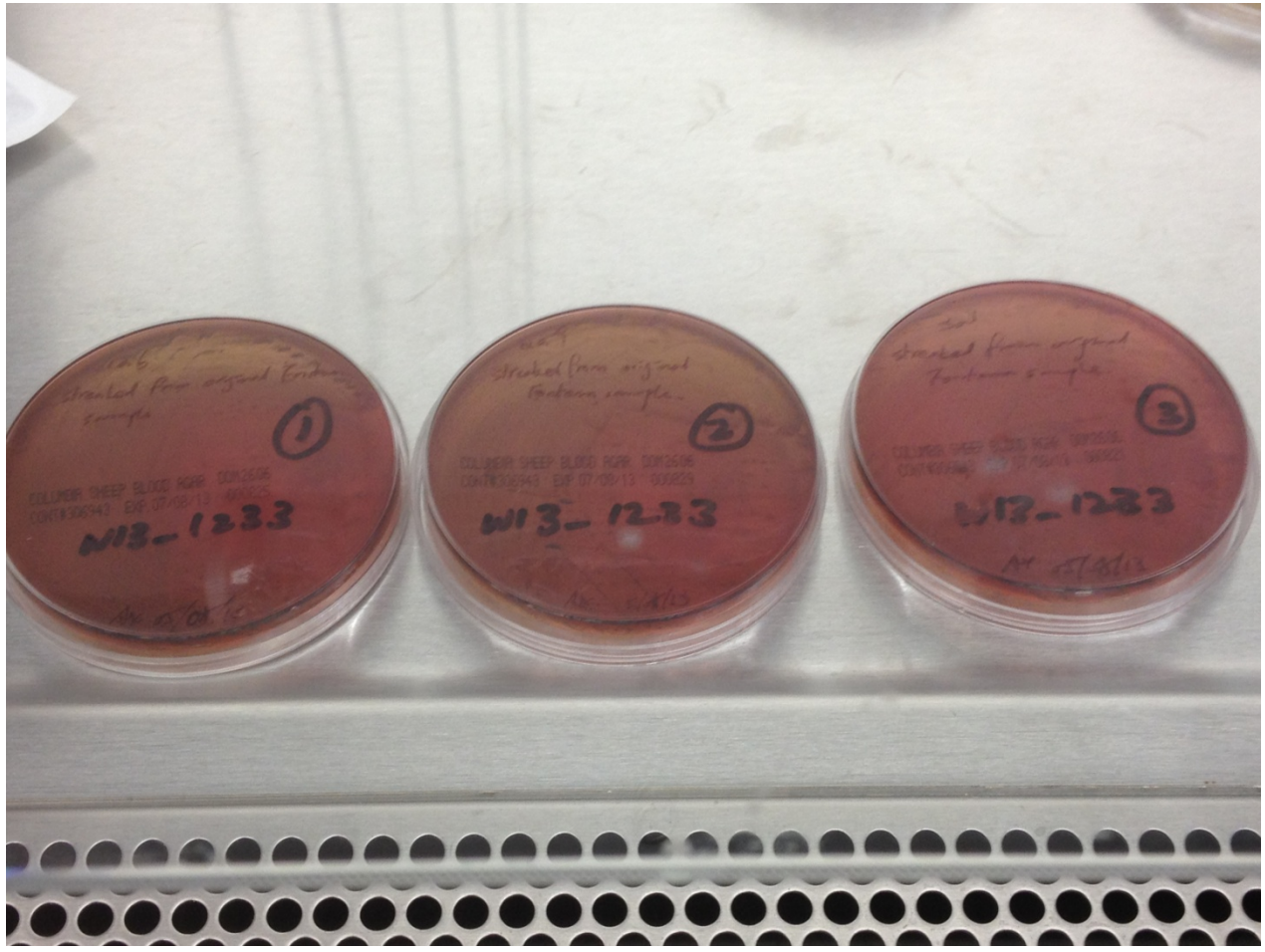


Figure 9.2: AgResearch streaked from original Fonterra sample (TSA plate 6/7/2013) and dated 5th August 2013. AHL accession numbers W13/1233 – 1-3. Photograph taken on 27 August 2013.

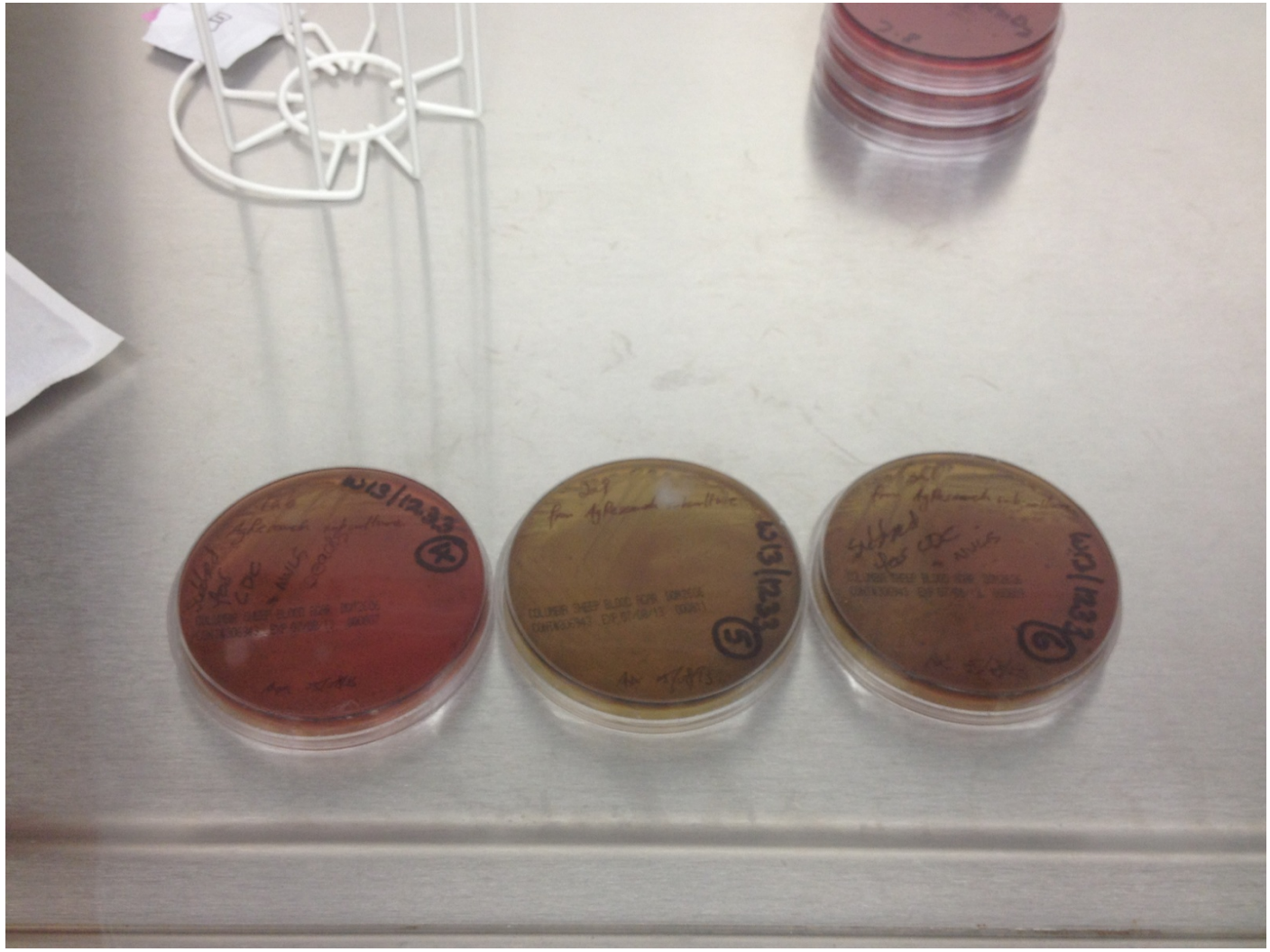


Figure 9.3: AgResearch subcultured from AgResearch subculture and dated 5/8/2013. AHL accession numbers W13/1233 4-6. Photograph taken on 27 August 2013.

10 Appendix: Traceability of laboratory testing at AHL on bacterial isolates

AHL Accession number	Details of bacterial cultures provided from AgResearch				AHL and contracted Laboratory testing (Generation number in red)						
	AgResearch/Fonterra Labels	Media	Date of subculture	Generation number	Bact id	16srRNA Seq	Toxin PCR	NGS	ELISA	CDC	NVSL
W13/1233 – 1	1a6 'streak from original (Fonterra sample)	BA	5/8/2013	**4°**		AgR sub 5/8	AHL sub 7/8 and 15/8 **5° and 6°**	AgR sub 5/8	AHL sub 7/8 **5°**	AHL sub 7/8 and 8/8 **5°**	AHL sub 8/8 and 9/8 **5°**
W13/1233 – 2	2a9 'streak from original (Fonterra sample)	BA	5/8/2013	**4°**	AHL sub 7/8 **5°**	AgR sub 5/8 AHL sub 7/8 **5°**	AHL sub 7/8 and 15/8 **5°**		AHL sub 7/8 **5°**	AHL sub 7/8 and 8/8 **5°**	AHL sub 8/8 and 9/8 **5°**
W13/1233 – 3	3a1 'streak from original (Fonterra sample)	BA	5/8/2013	**4°**		AgR sub 5/8	AHL sub 7/and 15/8 **5°**		AHL sub 7/8 **5°**	AHL sub 7/8 and 8/8 **5°**	AHL sub 8/8 and 9/8 **5°**
W13/1233 – 4	1a6 (AgResearch subculture)	BA	5/8/2013	**5°**			AHL sub 7/8 and 15/8 **6° and 7°**		AgR sub 5/8	AHL sub 7/8 and 8/8 **6°**	AHL sub 8/8 and 9/8 **6°**
W13/1233 – 5	2a9 (AgResearch subculture)	BA	5/8/2013	**5°**			AHL sub 7/8				
W13/1233 – 6	3a1 (AgResearch subculture)	BA	5/8/2013	**5°**	AHL sub 7/8 **6°**	AHL sub 7/8 **6°**	AHL sub 7/8 and 15/8 **6° and 7°**		AgR sub 5/8	AHL sub 7/8 and 8/8 **6°**	AHL sub 8/8 and 9/8 **6°**
W13/1233 – 7	(Cl.sporogenes 1a6)	Meat	6/7/2013	**3°**	AHL sub 7/8 **4°**	Font sub 6/7 AHL sub 7/8 **4°**	AHL sub 7/8 and 12/8 **4°**	AHL sub 12/8 **5°**	Font sub 6/7	AHL sub 7/8 and 8/8 **4°**	AHL sub 8/8 and 9/8 **4°**
W13/1233 – 8	Cl.sporogenes 2a9)	Meat	6/7/2013	**3°**			AHL sub 7/8 **4°**				
W13/1233 – 9	Cl.sporogenes 3a1)	Meat	6/7/2013	**3°**			AHL sub 7/8 **4°**				

11 Appendix Preliminary report and laboratory documents from AgResearch to Fonterra Co-operative Dairy Company Limited

[Refer separate files:
Appendix 11 AFLP results 7 Aug 2013.pdf
Appendix 11 AgResearch Mouse necropsies report 8 Aug 2013.pdf
Appendix 11 AgResearch Prelim Report for Fonterra- August 2013.pdf
Appendix 11 AgResearch Mouse Clinical signs and necropsies 27 July to 14 Aug 2013 .pdf]

[This appendix withheld from public release]

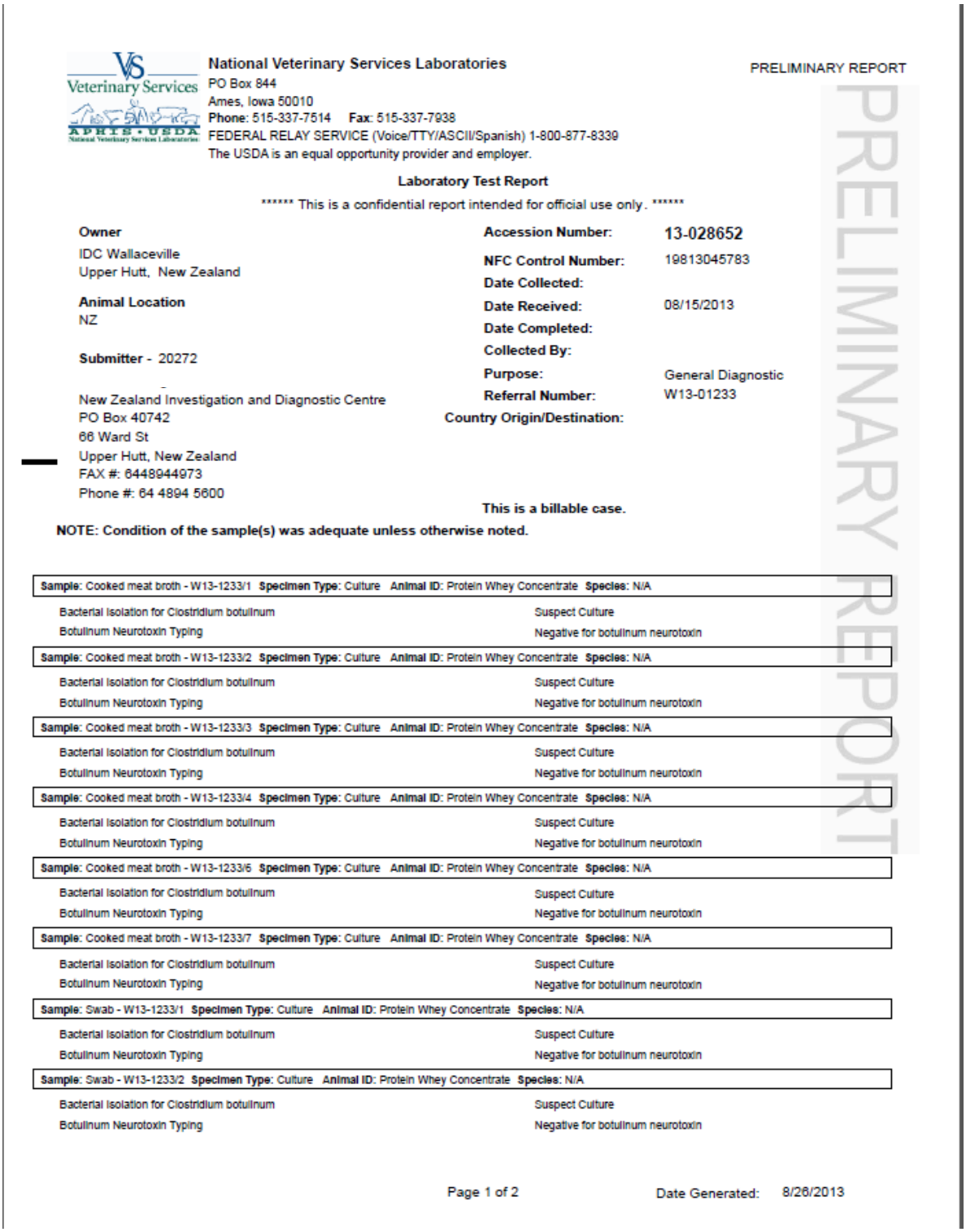
12 Appendix Fonterra summary report 3 08082013 and timeline

[Refer separate files:
Appendix 12 Fonterra Laboratory Sequence of Analysis V5_undated.docx
Appendix 12 Fonterra Summary Report 3 08082013.doc]

[This appendix withheld from public release]

13 Appendix NVSL, USDA Report

Appendix 13 USDA NVSL Preliminary Report 13-028652_1_26 Aug 2013.pdf



National Veterinary Services Laboratories
PO Box 844
Ames, Iowa 50010
Phone: 515-337-7514 **Fax:** 515-337-7938
FEDERAL RELAY SERVICE (Voice/TTY/ASCII/Spanish) 1-800-877-8339
The USDA is an equal opportunity provider and employer.

PRELIMINARY REPORT

Laboratory Test Report

****** This is a confidential report intended for official use only. ******

Owner
IDC Wallaceville
Upper Hutt, New Zealand

Animal Location
NZ

Submitter - 20272

New Zealand Investigation and Diagnostic Centre
PO Box 40742
66 Ward St
Upper Hutt, New Zealand
FAX #: 6448944973
Phone #: 64 4894 5600

Accession Number: 13-028652
NFC Control Number: 19813045783
Date Collected:
Date Received: 08/15/2013
Date Completed:
Collected By:
Purpose: General Diagnostic
Referral Number: W13-01233
Country Origin/Destination:

This is a billable case.

NOTE: Condition of the sample(s) was adequate unless otherwise noted.

Sample: Cooked meat broth - W13-1233/1 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Cooked meat broth - W13-1233/2 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Cooked meat broth - W13-1233/3 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Cooked meat broth - W13-1233/4 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Cooked meat broth - W13-1233/6 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Cooked meat broth - W13-1233/7 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Swab - W13-1233/1 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin
Sample: Swab - W13-1233/2 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A	
Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin

Page 1 of 2 Date Generated: 8/26/2013

PRELIMINARY REPORT

Accession: **13-028652** Owner: IDC Wallaceville Referral Number: W13-01233

Sample: Swab - W13-1233/3 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A

Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin

Sample: Swab - W13-1233/4 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A

Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin

Sample: Swab - W13-1233/6 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A

Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin

Sample: Swab - W13-1233/7 **Specimen Type:** Culture **Animal ID:** Protein Whey Concentrate **Species:** N/A

Bacterial Isolation for Clostridium botulinum	Suspect Culture
Botulinum Neurotoxin Typing	Negative for botulinum neurotoxin

All cultures were negative for botulinum neurotoxin by mouse bioassay after 7 days of anaerobic broth incubation. Pure growth of colonies consistent with Clostridium was obtained from plate culture from all samples at the time of broth inoculation, indicating all cultures were viable. DNA sequencing will be reported separately.

Results authorized by: Dr. , Bacterial Identification Section, Diagnostic Bacteriology Laboratory

Help Us Help You

(This new section will be updated periodically with tips for submitters.)

Please remember to submit payment or your User Fee account number at the time of submission. If you would like our billing department to contact you for a credit card number, please provide a valid contact number.

Page 2 of 2 Date Generated: 8/26/2013

PRELIMINARY REPORT

Appendix 13 USDA NVSL email RE Urgent testing World courier AWB 640013174_27 Aug 13.htm

[N.B. The xxxx in this email are redactions by MPI to protect the identity of individuals.]

From: **xxxxx, xxxxxxxx** - APHIS [xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Tuesday, 27 August 2013 8:46 a.m.
To: **xxxxxxxx** xxxxxx (xxxx); xxxxxx,xxxxxxx x - APHIS
Cc: **xxxxxxxx, xxxxx x** - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx; xxxxx xxxxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

xxxx,

You should have received a report by e-mail for accession 13-028652 marked as "preliminary" a few minutes ago. The report contains the final results for the mouse bioassay testing on these samples. The report is labeled as preliminary because sequencing is still in process. Once sequencing results are complete, you will receive a final report with both sets of results.

Let me know if you have any questions – I added a brief note summarizing the testing.

xxxx

xxxxxxxx xxxxx DVM
xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

From: xxxxxxx xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Thursday, August 22, 2013 4:34 PM
To: xxxxx, xxxxxxxx - APHIS; xxxxxx, xxxxxxx x - APHIS
Cc: xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx; xxxxx xxxxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

Thanks very much for the update, xxxx. Much appreciated.

xxxx

From: xxxxx, xxxxxxxx - APHIS [mailto:xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Friday, 23 August 2013 12:51 a.m.

To: xxxxxxx, xxxxxx (xxxx); xxxxxx, xxxxxxx x - APHIS
Cc: xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx; xxxxx xxxxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

xxxx,

The initial inoculations with the 24h incubated enrichments showed mild non-specific signs (depression, ruffling) which resolved within several hours. A very few mice showed mild wasp waist which also resolved. Because the signs were so non-specific, suggesting a sub-lethal amount of toxin or no toxin, we waited to perform further testing until the 5-10 day enrichment cultures from the swabs and broths were complete – those enrichments will be processed and inoculated this morning. If the isolates are toxogenic, we would anticipate typing the first isolate later today or tomorrow, and ideally performing the typing inoculations for the rest of the samples by the end of the week. We hope to have at least preliminary results by Friday (again if they are toxogenic), and if everything works as planned, final results for the toxin typing mid next week. We will make certain to update you once we have completed the first round of injections today.

The isolates were definitely viable, as we had growth on plates consistent with Clostridia. DNA has been extracted for sequencing.

xxxx

xxxxxxxx xxxxx DVM
xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

From: xxxxxx xxxxxx (xxxx) [mailto: xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Wednesday, August 21, 2013 10:46 PM
To: xxxxxx, xxxxxxx x - APHIS; xxxxx, xxxxxxxx APHIS
Cc: xxxxxxxx, xxxxx x - APHIS; xxxxx, xxxxx x - APHIS; xxxxxx xxxxxx; xxxxx xxxxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

Hi xxxxxxx and xxxxxxxx,

We were wondering if you could please supply us with any up-dates on the progress of testing of the protein whey concentrate samples.

I realize it is too early for a final report but an interim report describing any early results, if possible, would be much appreciated (e.g. the health or otherwise of the mice within the first 24 hrs following injection with the NZ isolates).

Are you able to provide us a rough date of we can expect a preliminary/final report on the mouse bioassay?

Thanks a lot for your help
xxxx

From: xxxxxx, xxxxxxx x - APHIS [mailto:xxxxxxx.x.xxxxxx@aphis.usda.gov]
Sent: Tuesday, 13 August 2013 8:51 a.m.
To: xxxxxxx xxxxxx (xxxx); xxxx, xxxxxxxx - APHIS
Cc: xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx, x - APHIS; xxxxxx xxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

xxxx,
Genome sequencing is a possibility but depends specifically on what you are looking for. Is it safe to assume you are not talking about a fully closed and annotated genome but rather conducting genome sequencing and alignment with a reference strain? Let me know a little more about what you are looking for with sequencing and I can better discern how much help we could offer. Our current pipeline is mostly Illumina MiSeq sequencing followed by SNP analysis for epidemiology purposes. We don't currently have our own database of Clostridium though so would be relying on aligning to previously published references strains in public databases or a sequence that you would provide.

Regards,

Xxxxxxx x xxxxxx, DVM, PhD
Xxxxxxxx, xxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxx
National Veterinary Services Laboratories
1920 Dayton Ave, Ames, IA 50010
xxx-xxx-xxxx
xxxxxxx.x.xxxxxx@aphis.usda.gov

From: xxxxxxx xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Monday, August 12, 2013 3:36 PM
To: xxxx, xxxxxxxx - APHIS
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

Thank goodness, xxxxxxxx, it has passed inspection by both FDA and USDA. Now just has to clear customs.

Another question please. Does your lab do full genome sequencing of the Clostridium genome? If not, do you know of, or are you able to forward, DNA to another USA lab that does such sequencing?

Regards
xxxx

From: xxxxx,xxxxxxxx - APHIS [mailto:xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Tuesday, 13 August 2013 12:38 a.m.
To: xxxxxx xxxxx (xxxx)

Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

xxxx,

I haven't seen any communication from the FDA yet, but we will keep you posted.

Xxxxxxxx xxxxx DVM
Xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

From: xxxxxxxx xxxxxx (xxxx) [mailto:xxxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Sunday, August 11, 2013 4:29 PM
To: xxxxx, xxxxxxxx - APHIS
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx
Subject: RE: Urgent testing World courier AWB 640013174

Hi xxxxxxxx and xxxxxxx,

The samples are in San Francisco on FDA hold. Apparently the shipment can be held without explanation for up to 5 days. However, any communication by the FDA will be with you the consignee, and therefore we would appreciate receiving any updates that you get.

Thank you
xxxx

From: xxxxx, xxxxxxxx - APHIS [mailto:xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Saturday, 10 August 2013 2:56 a.m.
To: xxxxxxx xxxxxx (xxxxk)
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx
Subject: RE: Urgent testing

xxxx,

Sounds good –

Are the cooked meat broth and cultures the same 6 isolates? Are the broths pure culture or the original enrichments, and how long were they incubated? In other words, if we start by testing the broths immediately and get an ID from those, do we need to type the cultures as well once the enrichments are complete, or are they essentially the same thing?

We'll get them set up as quickly as possible and send preliminary results as soon as we can get them.

xxxxxxxx xxxxx DVM
xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

From: xxxxxxx xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Thursday, August 08, 2013 11:34 PM
To: xxxxx,xxxxxxxx - APHIS
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS; xxxxxxx xxxxxx
Subject: RE: Urgent testing

Hi xxxx,

Today World Courier (AWB 640013174) picked up 6 x Cultures in cooked meat broth and 6 x Cultures on transport swabs to be shipped at refrigeration temperature under controlled conditions. They will probably arrive your time Monday.

They are accompanied by a submission form but we would appreciate botulinum neurotoxin typing (A to F). As this testing is very urgent we did not have time to arrange for frozen medium preparation.

I will leave it up to you to most efficiently test for toxins as you think best, although initial confirmed ID of at least 1 sample would be ideal (noting your lack of mice).

Thanks a lot

xxxx

From: xxxxx,xxxxxxxx - APHIS [mailto:xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Thursday, 8 August 2013 1:52 a.m.
To: xxxxxxx xxxxxx (xxxx)
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS
Subject: RE: Urgent testing

xxxx,

We are able to type for types A-F botulinum. The antisera for type G is no longer available from our source.

We do not have enough mice available to immediately screen and fully type all 10 of the samples. If they are not confirmed as toxogenic, I would propose first screening all 10 samples. If the samples are toxogenic, we would then either type one sample with A-B-E, C,

D, and F or type that one with A-F (skipping trivalent A-B-E and going straight to individual antitoxins). We could then type an additional two samples with our remaining mice. At that point we would need to wait until we got more mice to complete testing, but we could issue a preliminary report. On the other hand, if you have a preliminary identification of toxin type based on sequencing or PCR, we could screen and type for that toxin all at once, and we would have enough mice to do all 10 samples. We may also be able to send DNA to another US lab that does a screening PCR for types A, B, and C to see if we can narrow what toxin type to test for.

If they are found to be toxogenic prior to shipping, you would need whatever New Zealand requires for an export permit, and we require a permit from the national select agent registry called a CDC Form 2. The permit must be issued for each shipment and is good for 30 days from date of issuance. It typically takes about a week to have one issued and there is no cost associated, so I would suggest we go ahead and initiate that permit, even if you have not confirmed the cultures as toxogenic yet. I attached the fillable PDF. Please fill out section B and send back to me, and I will fill in A and get the application submitted as quickly as possible. You can ignore section 14 and the part about submitting a form 4 on #23 – that's for US shippers only. If the full address won't fit (It's set up for US addresses), just include the full address in the body of the e-mail, and we will handwrite the remaining information in when we submit the form.

xxxx

xxxxxxxx xxxxx DVM
xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

From: xxxxxxx xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Tuesday, August 06, 2013 8:24 PM
To: xxxxx, xxxxxxxx - APHIS
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS
Subject: RE: Urgent testing

Thanks for the information xxxxxxxx.

There would be about 10 samples to test.

What neurotoxins are you able to type by the bioassay?

Out of interest and due to possible urgency, if the cultures were shown to be a toxigenic strain, what additional permits etc would be required and are these currently available at NVSL?

Regards
xxxx

From: xxxxx, xxxxxxxx - APHIS [mailto:xxxxxxxx.xxxxx@aphis.usda.gov]
Sent: Tuesday, 6 August 2013 4:37 a.m.
To: xxxxxxx xxxxxx (xxxx)
Cc: xxxxxx, xxxxxxx x - APHIS; xxxxxxxx, xxxxx x - APHIS; xxxxxx, xxxxxx x - APHIS
Subject: FW: Urgent testing

xxxx,

That permit is acceptable for cultures as long as they have not been confirmed as toxogenic *C. botulinum*. We don't frequently get suspect cultures for botulinum, but my suggestion for shipping would either be as a frozen culture in any standard freezing medium shipped in plastic cryovials shipped on dry ice, or on a heavily inoculated swab in an anaerobic transport medium at approximately 4 degrees C (packed with ice packs separated by packing material). The anaerobic transport medium would be ideal if you have it available and there are no shipping delays, but the frozen cultures should survive transport as well.

How many cultures are there? Are they from the same source, so that we could potentially pool them? We do not have mice on hand at the moment but another lab on our campus has some that we can use. Depending on the number of samples, or if we can pool the culture enrichments if they came from the same original sample, we may be able to complete the testing relatively quickly (for the mouse bioassay) – at least 5 days for enrichment plus up to 5 days for the mouse bioassay – if we have to wait until we receive an additional batch of mice to complete testing, it will likely be 3-5 weeks. If you have enrichments in anaerobic medium already, we could potentially try testing directly from those, reducing the test time by several days.

If the test is negative, it would indicate the culture is either *C. sporogenes* or non-toxogenic *C. botulinum*.

Do you have an idea of when you would ship the samples? As usual, please send a tracking number upon shipping to xxxxxx and me so that we can notify our sample processing department.

Thanks,

xxxx

xxxxxxxx xxxxx DVM
xxxxxxxxxx xxxxxxx xxxxxxx
USDA APHIS NVSL DBL-BI
1920 Dayton Ave.
Ames, IA 50010
Phone: xxx-xxx-xxxx
Cell: xxx-xxx-xxxx
xxxxxxxx.xxxxx@aphis.usda.gov

believe you have received this message in error, please notify the sender and delete the email immediately

From: xxxxxxx xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Sunday, August 04, 2013 8:57 PM
To: xxxxxx, xxxxxxx x - APHIS
Subject: Urgent testing

Hi xx xxxxxx,

We have some cultures isolated from a bovine milk by-product (Whey Protein Concentrate) of NZ origin that we would like tested/typed urgently by the C. botulinum mouse bioassay. The bacteria have been presumptively cultured as C. botulinum/C. sporogenes (not definitively speciated yet).

Do you think your USDA import permit 41429 Research covers such cultures (1. Various animal and avian microorganisms and/or disease agents for identification)?

If not, how long would it take to produce another permit?

What form of culture medium, shipping condition etc would you like the isolate sent ?

Thanks a lot
xxxx

xx xxxxxxx xxxxxx BVSc MSc PhD | xxxxxx xxxxxxxxx- xxxxxxxxxx | Investigation and Diagnostic Centres & Response | Compliance & Response| Ministry for Primary Industries | 66 Ward St | PO Box 40-742 | Upper Hutt 5140 | New Zealand
Telephone: xx-x-xxx xxxx | Facsimile: xx-x-xxx xxxx | Mobile: xxx-xxx xxxx | Web: www.mpi.govt.nz

14 Appendix CDC Report

Appendix 14 RE CDC email re testing on NZ cultures_28 Aug 2013.htm

[N.B. The xxxx in this email are redactions by MPI to protect the identity of individuals.]

From: **xxxxxxx, xxxxx** (CDC/OID/NCEZID) [xxxx@cdc.gov]
Sent: Wednesday, 28 August 2013 4:51 a.m.
To: **xxxxx xxxxxxxx**
Cc: **xxxxxxx xxxxxx (xxxx)**; xxxxxxxx xxxxxxxxxxxx; xxxxxxx xxxxxxx; xxxxxx- xxxxx, xxxxx (CDC/OID/NCEZID); xxxxxx, xxxxxxxx (CDC/OID/NCEZID); xxxxxxxxxxx, xxxxxxxxxx (CDC/OID/NCEZID)
Subject: RE: CDC testing on NZ cultures

Xxxxx and xxxx,

Based on the Final mouse bioassay test results today, CDC has found no evidence of the presence of a botulinum neurotoxin producing species of *Clostridium* in any of the cultures submitted to us.

I will be happy to provide you with a written report sometime next week.

Susan

xxxxx xxxxxxxx, PhD
xxxx xxxx
National Botulism Laboratory Team
CDC

From: xxxxxxxx, xxxxx (CDC/OID/NCEZID)
Sent: Thursday, August 22, 2013 7:37 PM
To: 'xxxxx.xxxxxxxx@mpi.govt.nz'; xxxxxx, xxxxxxxx (CDC/OID/NCEZID)
Cc: 'xxxxxxx.xxxxxx@mpi.govt.nz'; 'xxxxxxxx.xxxxxxxxxxxx@mpi.govt.nz'; 'xxxxxxx.xxxxxx@mpi.govt.nz'; xxxxxx-xxxxx, xxxxxx (CDC/OID/NCEZID)
Subject: Re: CDC testing on NZ cultures

xxxxx,

We received 12 samples. 6 were swabs in anaerobic transport medium and 6 were liquid cultures.

On receipt we transferred each swab into a fresh chopped meat broth. We incubated these for 5 days under anaerobic conditions. We initiated mouse assay on the 5 day cultures on Tuesday. At 48 hours, there is no evidence of the presence of a botulinum toxin producing Clostridium sp in any of the 6 cultures. This is a preliminary result; we will be able to provide a final result on Tuesday next week.

We innoculated egg yolk agar plates with each of the broth cultures sent to us. After incubation under anaerobic conditions, 2 suspect colonies (morphology consistent with

Clostridium sp) from each plate were each innoculated into cooked meat broth. These 12 cultures (2 representative colonies per original broth culture submitted) were incubated 5 days under anaerobic conditions. All 12 cultures were tested by mouse bioassay; animals injected on Wednesday. At 24 hours, there is no evidence of the presence of a botulinum toxin producing Clostridia sp in any of the 12 cultures. This is a preliminary result. Final results will be available next Tuesday.

Assuming there is no change in the results (Negative) through Monday next week, we have no further plans for testing.

xxxxx

From: xxxxx xxxxxxxx [mailto:xxxxx.xxxxxxxx@mpi.govt.nz]
Sent: Wednesday, August 21, 2013 08:26 PM Eastern Standard Time
To: xxxxxxxx, xxxxx (CDC/OID/NCEZID); xxxxxx, xxxxxxx(CDC/OID/NCEZID)
Cc: xxxxxxx xxxxxx (xxxx) <xxxxxxx.xxxxxx@mpi.govt.nz>; xxxxxxxx.xxxxxxxxxxx <xxxxxxxx.xxxxxxxxxxx@mpi.govt.nz>; xxxxxxx xxxxxx <xxxxxxx.xxxxxx@mpi.govt.nz>
Subject: CDC testing on NZ cultures

Dear xxxxx and xxxxxxxx,

I hope you don't mind me contacting you instead of xxxx, but xxxx is away ill at the moment. Sorry, quite a few questions below, but any early information you can provide would be greatly appreciated.

- Are you able to share any early results with us or an indication of the health or otherwise of the mice within the first 24 hrs following injection with the NZ isolates?

- Are you able to provide us with an update on your timeframe for when we can expect a preliminary/final report on the mouse bioassay and ELISA?

- Also, one of my MPI colleagues has asked a question whether the mouse bioassay will be staged or repeated and if so what is the likelihood of obtaining a different result?

Thank-you both very much for helping us.

Cheers
xxxxx

Xx xxxxx xxxxxxxx | xxxxxx xxxxxxx, xxxxxx xxxxxx xxxxxxxxxx
Investigation and Diagnostic Centres and Response | Compliance and Response
Ministry for Primary Industries - Manatū Ahu Matua | 66 Ward Street | PO Box 40742 | Upper Hutt | New Zealand
Telephone: xx-x-xxx xxxx | Facsimile: xx-x-xxx xxxx | Mobile: xxx-xxx xxxx| Web: www.mpi.govt.nz

From: xxxxxxx xxxxxx
Sent: Tuesday, 20 August 2013 5:43 p.m.
To: xxxxxxxx, xxxxx (CDC/OID/NCEZID); xxxxxx, xxxxxxxx (CDC/OID/NCEZID)
Cc: xxxxxxx xxxxxx (xxxx); xxxxxxxx xxxxxxxxxxx; xxxxx xxxxxxxx
Subject: RE: urgent request

Hi xxxxx,

We will follow up with xx xxxxxx at FDA regarding this request. In the meantime we have a teleconference with the FDA in the morning (Wed am NZ time) although this isn't with xx xxxxxx. However, we will also follow up with them regarding this request.

Best wishes,
xxxx
From: xxxxxxxx, xxxxx (CDC/OID/NCEZID) [mailto:xxxx@cdc.gov]
Sent: Tuesday, 20 August 2013 4:55 a.m.
To: xxxxxxx xxxxxx; xxxxxx, xxxxxxxx (CDC/OID/NCEZID)
Cc: xxxxxxx xxxxxx (xxxx); xxxxxxxx xxxxxxxxxxx
Subject: RE: urgent request

xxxx,

I have been asked by US FDA if we can share the cultures that were sent to us.

Is it ok for us to do this?

If you want to contact FDA directly, you can contact xx. Xxxxxx xxxxxx, CFSAN/FDA at xxxxxx.xxxxxx@fda.hhs.gov

xxxxx

From: xxxxxxx xxxxxx [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Monday, August 05, 2013 8:07 PM
To: xxxxxx, xxxxxxxx (CDC/OID/NCEZID); xxxxxxxx, xxxxx (CDC/OID/NCEZID)
Cc: xxxxxxx xxxxxx (xxxx); xxxxxxxx xxxxxxxxxxx
Subject: RE: urgent request
Importance: High

Dear xxxxxxxx,

thank you very much for taking to time to speak with us earlier regarding the suspect isolates we would like to send to you for *Cl. botulinum* testing by both the mouse bioassay and ELISA for all toxin serotypes. As indicated we wish to send these samples to you as soon as possible. Ideally we would like to do this our Wednesday but we will keep you posted on this. Please note we will send the samples via World Couriers both as frozen cultures in cooked meat broth and on swabs in transport media chilled. Please can you confirm these are suitable samples for your testing, also please can you confirm this will provide you with sufficient sample to be able to perform both assays concurrently? One additional question I had is if samples arrive on your Thursday or Friday this week will they be immediately tested (i.e. are you open over the weekend) or will testing be started next week?

As mentioned on the phone earlier we would also like an international expert to review some testing that has been performed on suspect isolates by another laboratory. Xx xxxxxxxx would you be willing to assist with this and if so what time frame would be feasible with you? I will provide a terms of reference later today for your consideration that will detail what service we are requesting. Obviously we will pay whatever charge is necessary to cover your time. If this isn't feasible for you please can you recommend someone else we could approach?

xxxxxxxx I hope we have provided sufficient information to obtain approval of the permittee on the import permit to send these samples at the earliest opportunity. If you do require any

further information please let me know as soon as possible as it is very urgent for us to get these samples to you this week.

Again as indicated earlier on the phone it would be greatly appreciated if we could touch base with one of you our tomorrow (Wed) morning which will be your Tuesday afternoon. xxxxxxxx I understand you are busy with training until 17:00, and xx xxxxxxxx I understand you depart work at 15:30 but would you be willing to speak to us on the phone at 15:00 on Tuesday your time? If so please can you provide a phone number. If this isn't feasible xxxxxxxx would we be able to speak to you briefly after your training?

Thank you for your assistance in facilitating this testing, I hope to speak to you soon.

Kind regards,
xxxx

xxxxxxx xxxxxx PhD | xxxx xxxxxxx, xxxxxxxxxx | Compliance and Response | Investigation and Diagnostic Centres and Response | Ministry for Primary Industries | 66 Ward St | PO Box 40-742 | Upper Hutt | New Zealand
Telephone: xx-x-xxx xxxx | Facsimile: xx-x-xxx xxxx | Web: www.mpi.govt.nz

From: xxxxxxx xxxxxx (xxxx)
Sent: Tuesday, 6 August 2013 11:05 a.m.
To: xxxxxxx xxxxxx
Subject: FW: urgent request

From: xxxxxx, xxxxxxxx (CDC/OID/NCEZID) [mailto:xxxx@cdc.gov]
Sent: Tuesday, 6 August 2013 7:35 a.m.
To: xxxxxxx xxxxxx (xxxx)
Subject: RE: urgent request

Dear xx xxxxxx,

Please find attached the import permit to include with the package.

This is our address:

STAT (Attn: Botulism Lab)
Centers for Disease Control and Prevention
1600 Clifton Rd NE
Atlanta, GA 30329
USA

If you need a point of contact for the shipment, please indicate:
Xxxxxxxx xxxxxx
Phone: (xxx) xxx-xxxx

I've also attached the reprint you requested. Unfortunately, the ELISA kits are not commercially available.

Best regards,

xxxxxxxx xxxxxx, PhD
xxxxxxxx xxxxxxxx xxxxxxxxxxxxxx xxxx
National Botulism Laboratory Team
Centers for Disease Control and Prevention
E-mail: xxxxxxx@cdc.gov

Phone: (xxx) xxx-xxxx

From: xxxxxxxx, xxxxx (CDC/OID/NCEZID)
Sent: Monday, August 05, 2013 6:55 AM
To: xxxxxx, xxxxxxxx (CDC/OID/NCEZID)
Subject: FW: urgent request

From: xxxxxxx,xxxxxx (xxxx) [mailto:xxxxxxx.xxxxxx@mpi.govt.nz]
Sent: Sunday, August 04, 2013 9:11 PM
To: xxxxxxxx, xxxxx (CDC/OID/NCEZID)
Subject: urgent request

Dear xx xxxxxxxx,

I was reading your 2011 paper on botulinum toxin bioassay in Comp. Med, 61, 235-242, and was wondering if you could please refer me to the appropriate person at CDC, Atlanta.

I am a scientist at the national diagnostic lab in New Zealand and we have some very urgent suspect C. botulinum or C. sporogenes cultures (not yet speciated) that we would like the toxin identified by C. botulinum mouse bioassay.

Therefore, we would much appreciate

1. A contact person
2. The correct shipping address
3. An import permit. The culture was isolated from a bovine milk by-product (Whey Protein Concentrate) of NZ origin. The permit would need to cover microorganisms.

Thanks very much for your time
xxxx

xx xxxxxxx xxxxxx BVSc MSc PhD | xxxxxx xxxxxxxxxt - xxxxxxxxxxy | Investigation and Diagnostic Centres & Response | Compliance & Response| Ministry for Primary Industries | 66 Ward St | PO Box 40-742 | Upper Hutt 5140 | New Zealand

Telephone: xx-x-xxx xxxx | Facsimile: xx-x-xxx xxxx | Mobile: xxx-xxx xxxx | Web: www.mpi.govt.nz