Usefulness of testing for *Clostridium botulinum* in powdered infant formula and dairy-based ingredients for infant formula

**Dated: 27 August 2013**

### Background

In early August 2013, a New Zealand dairy company announced that a whey protein concentrate it produces for infant formula, beverages and animal feed was potentially contaminated with *Clostridium botulinum* (*C. Botulinum*), leading to recalls of infant formula products. Although there have been no reports of any public health issues from this incident, it has raised the level of concern for the safety of these products with respect to *C. botulinum*. The International Commission on the Microbiological Specifications for Foods (ICMSF) ([www.icmsf.org](http://www.icmsf.org)) has received a number of enquiries from industry and regulatory authorities to provide advice on the appropriateness of testing for *C. botulinum* in these products. In response to these enquiries, the ICMSF has prepared this document.

### Clostridium botulinum

*C. botulinum* is an important foodborne pathogen that causes serious neuroparalytic disease. The organism comprises four physiologically distinct groups capable of producing protein neurotoxins that have similar toxicological activity. The organism is an anaerobic, gram-positive, spore-forming bacterium that is commonly found in soils throughout the world. There are four clinically distinct forms of botulism in humans: foodborne botulism (resulting from ingestion of pre-formed toxin in a food); infant botulism (as a result of colonisation of the infant intestine accompanied by toxin production *in vivo*); adult infectious botulism (similar to infant botulism but occurring in immune-compromised adults); wound botulism (caused by the organism infecting deep wounds and producing toxin *in vivo*). Human botulism is caused by types A, B, E, and rarely F and there are rare reports of some strains of non-botulinum clostridia (*e.g. C. butyricum and C. baratii*) also causing botulism. Due to the serious nature of the neuroparalytic disease caused and unparalleled potency of the toxin, *C. botulinum* remains one of the major targets for control in foods today.

### Product use and risks

Due to its potency, there is no tolerance for the presence of neurotoxin or for conditions allowing growth of *C. botulinum* in foods. For foods for which *C. botulinum* is a relevant hazard to consider, industry assures the safety by eliminating the hazard or by controlling its presence or possible outgrowth. The primary factors for control of *C. botulinum* in foods are prevention of growth through reduced pH (<4.6) or reduced water activity (<0.93, also achieved by storing foods frozen), through the presence of preservatives or through a combination of these, or alternatively thermal inactivation (121°C for 3 min for the most heat resistant types in low acid foods) to effectively eliminate the organism from products. The main risks for foodborne botulism are associated with inadequate control of one or more of these main factors, leading to the survival, germination and outgrowth of *C. botulinum* spores. In many countries, foods associated with foodborne botulism tend to be home-prepared, such as traditionally-prepared salted or fermented foods (*e.g. fish, cured meats*), and foods canned or bottled in the home. In these cases, foods are prepared and then stored under conditions that allow outgrowth of spores that are present in the raw materials. Occasionally, commercially manufactured products can cause foodborne botulism, where again, there is failure in applying the relevant controlling factors, such as inadequate heat treatment, inadequate storage temperature or inadequate control of water activity, allowing spores present in the raw materials to germinate and multiply in the food prior to consumption.
Presence of low levels of \textit{C. botulinum} spores in foods and the environment occur routinely, and with the exception of infants younger than 12 months (being the consumers particularly at risk for infant botulism) and specific highly susceptible adults (potential for adult infectious botulism), these spores are generally ingested with no apparent harm. The annual number of cases in most countries is very low in comparison to most other foodborne diseases, being in the order of 10s or 100s per year (Hauschild and Dodds, 1993). Dairy products have only very rarely been associated with outbreaks of \textit{C. botulinum} and less than 10 cases, mainly due to contaminated cheeses, have been reported in the course of the last decades (Lindström et al., 2010).

\section*{Infant botulism and infant formula}

Of the different forms of botulism, infant botulism is the most common form reported in the USA (Shapiro et al., 1998). Infant botulism is caused when neurotoxigenic \textit{Clostridium} spores are ingested, germinate and colonise the immature gut of the infant and produce neurotoxin. Infants less than one year are at risk of infant botulism and those less than 6 months are particularly vulnerable, which likely reflects the inability of the infant’s immature intestinal microflora to resist colonization by \textit{C. botulinum}. The clinical presentation and management of the disease is adequately discussed elsewhere (Radsel et al., 2013). The identification of foodstuffs associated with cases of infant botulism is difficult due to the generally long incubation time before manifestation of the disease, but the main documented evidence exists for honey as a vehicle (Midura, 1996) and spores of \textit{C. botulinum} have been isolated from honey (Hauschild et al., 1988, Al-Waili et al., 2012). In the UK, a case of infant botulism was presumably linked to infant formula (Brett et al., 2005) and reportedly \textit{C. botulinum} type B spores were found in both an opened and unopened can. However, the AFLP (Amplified Fragment Length Polymorphism) type of the isolate from the unopened can was different to that of the isolate from the infant. In addition, differences in the PCR profile of \textit{C. botulinum} isolated from the unopened can versus the isolate from the infant suffering from infant botulism led Johnson et al. (2005) to conclude that the infant formula powder was not the source of transmission of spores to the infant. With respect to hazardous microorganisms associated with infant formula, FAO/WHO categorized \textit{C. botulinum} as “causality less plausible or not yet demonstrated”, because although having been identified in powdered infant formula, they had not been implicated as causing illness in infants (FAO/WHO, 2004). Consequently, \textit{C. botulinum} is not considered a hazard in the Codex international hygiene standard for infant formula (CAC, 2008). In a recent report on infant botulism by the UK Food Standard Agency (ACMSF, 2006), experts carrying out a risk assessment on infant botulism agreed that, based on available data on the presence of spores of \textit{C. botulinum} in infant food (so broader than just infant formula) the most likely mean value was 0.3 spores per kg, the minimum mean was 0.001 spores per kg, and the maximum mean value was 10 spores per kg. The experts considered in their conclusions that even levels of 10 spores per pack of 121 g (hence <0.1 spore per serving of 13g) would only represent a low risk of disease.

\section*{Limitations of testing for \textit{Clostridium botulinum} in food or food ingredients}

Detection and enumeration of \textit{C. botulinum} in foods is difficult. This is partly because of the large differences in the microbial ecology of strains of the ‘species’. In fact, \textit{C. botulinum}, as currently defined, includes organisms that are sufficiently genetically different that they could be classified as distinct species (Fischer et al., 2012; Peek et al., 2010), and the species is mostly characterised by the ability to produce botulinum neurotoxins. Other \textit{Clostridium} species that produce botulinum toxins are now also recognised and multiple methods are required to detect all strains of relevance (Fischer et al., 2012). Methods based on enrichment, followed by confirmation by PCR, are generally recommended.
Confirmation of toxin production, using the mouse bioassay, is required in some situations (Solomon and Lilly, 2001), but this is not suited to routine food microbiology laboratories since special security and biosecurity precautions are required (EFSA, 2005). Therefore, only a few, specialised, laboratories internationally are able to do this work. Even though the mouse bioassay is the ‘gold standard’ method for detection of botulinum toxin, it has several drawbacks including non-specific mortality during the course of the test (often due to the response of the animals to endotoxins from Gram-negative bacteria), availability of appropriate controls and materials, long testing time (4 days), and animal ethics issues (AOAC international, 2001).

While testing for *C. botulinum* may be required in some circumstances, e.g., to assess a suspect lot, as part of a survey for a risk assessment, etc., routine testing of foods for presence of *C. botulinum* is not usually recommended. This consensus view is due both to the difficulty of testing for *C. botulinum* and also the very low levels of contamination of foods with this microorganism. For example, honey involved in cases of infant botulism has been found to contain as few as 5 - 80 spores per gram (Midura *et al.*, 1979; Arnon *et al.*, 1979). In others studies, levels of *C. botulinum* in honey that were not associated with infant botulism were not higher than 7 per 25 g (ACMSF, 2006). Notably, an oft-cited “infectious dose” for infant botulism is 10 - 100 spores (Arnon, 1992).

**Testing for Clostridium botulinum in powdered infant formula**

Whilst *C. botulinum* generally presents a low level of risk in powdered dairy products, spores of the organism have incidentally been found.

In a survey of dairy powders in France, levels in the range 2 - 5 per kg were reported (Carlin *et al.*, 2004) but were not known to be associated with illness. Barash *et al.* (2010) found very low levels of clostridial spores (1.1, > 23/100g) in powdered formulae but *C. botulinum* was not detected in the samples analysed.

To design a suitable sampling plan for testing, the required performance of the sampling plan needs to be decided on, i.e. what level of spores should be the basis for the microbiological criterion to be used to separate acceptable from unacceptable product lots. For typical infant formula, 12 - 14 g is made up in 100 ml of water, which is a common serving size of ‘reconstituted milk’. From the reports cited above, and for illustrative purposes only, it could be assumed that 1 spore per 100 g of powdered formula or for instance its main protein component whey powder has a low probability of causing infant botulism. A suitable sampling plan would then be one that provides confidence that if this level is exceeded it will be reliably detected.

The performance of sampling plans can be approximated by the binomial distribution function (Jarvis, 2008). A more sophisticated analysis has been presented by van Schothorst *et al.* (2009).

The binomial function permits a first estimate of the number of samples that must be tested and shown not to be contaminated, to provide assurance that the batch is not more contaminated than the microbiological criterion required, assuming that the contamination is homogenous throughout the batch. As there is some element of chance involved in any sampling scheme, the number of samples required will increase when higher levels of confidence are required. The number of samples will also depend on the size of the sample that is analysed.

Based on these considerations and assumptions, Table 1 exemplifies the number of samples that should be tested (at selected levels of confidence, and for different samples sizes) and shown not to be contaminated to be confident that the level of contamination in the lot is less than one spore per 100 grams of infant formula powder.
Table 1: Numbers of samples (of specified size) that must be tested from a lot (for a homogenous contamination) to ensure that the contamination with spores of *C. botulinum* is less than 1 per 100 g. The Table shows the effect of sample size and rigour of testing (expressed as confidence that the result is accurate).

<table>
<thead>
<tr>
<th>sample size</th>
<th>confidence in result of testing</th>
<th>number of samples required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 gram</td>
<td>95%</td>
<td>299</td>
</tr>
<tr>
<td>10 gram</td>
<td>95%</td>
<td>29</td>
</tr>
<tr>
<td>25 gram</td>
<td>95%</td>
<td>11</td>
</tr>
<tr>
<td>25 gram</td>
<td>99%</td>
<td>17</td>
</tr>
<tr>
<td>25 gram</td>
<td>90%</td>
<td>9</td>
</tr>
</tbody>
</table>

However, the level of contamination is unlikely to be homogenous throughout the batch, and it is more likely to be log-normally distributed. As will be discussed later, if it is assumed that the variability in contamination levels within a batch has a standard deviation of 0.8 (log[CFU/g]), a batch that satisfies the criterion of ≤1 spore/100g in 99% of cases, would have an average concentration throughout the batch of 1 spore per ~7.3 kg of powdered infant formula. No practical sampling scheme would be able to detect such low levels of contamination.

**Current practice and testing for sulphite reducing clostridial spores**

Given the methodological limitations described above, routine microbiological testing for *C. botulinum* to ensure food safety is not recommended. Instead, tests for sulphite-reducing clostridia (Fischer *et al.*, 2012) have been proposed to identify product lots produced under conditions that could be associated with a potential risk for *C. botulinum* when present.

The detection of sulphite-reducing clostridia (SRC) has been used extensively in both water and food microbiology, but their use is based on different assumptions and protocols. In water microbiology, SRC are largely used as either a faecal and/or process control indicator. The presence of SRC in the absence of other faecal indicators is indicative of remote or intermittent contamination, usually *Clostridium perfringens*, in the distant past (Sartory *et al.*, 1993; AS/NZS, 2000; Robles *et al.*, 2000; Adcock and Saint, 2001; Marcheggiani *et al.*, 2008). SRC use as a process control indicator is based on the greater resistance of *Clostridium* species to water treatments than viruses and protozoa (Adcock and Saint, 2001).

In food microbiology, use of SRC as a microbial indicator has been discussed for more than 50 years, but its routine use is limited to a relatively small number of foods (Mossel, 1959; Romagnoli and Brezzi, 1960; Prevost *et al.*, 2013). While originally proposed as an index test for pathogenic *Clostridium* (Mossel, 1959; Weenk *et al.*, 1991), this goal has been elusive due to different sources of *Clostridium* in foods (e.g. *C. perfringens* related to faecal contamination; *C. botulinum* related to soil contamination). Its current application in foods is either as an indication of faecal contamination (*C. perfringens*) and/or as an indicator of sanitation/process control related to potential growth and survival of anaerobic spore-forming bacteria. Standard methods for SRC are available for instance through ISO 15’213 (ISO, 2003) and US FDA (US FDA, 2001). There are differences in standard SRC methods currently used (e.g., FDA BAM vs. ISO) on
whether to heat food samples to eliminate vegetative cells. If a heat treatment is not used, confirmation is required due to the ability of a number of non-spore forming bacteria (e.g., *Proteus*, *Salmonella*) to reduce sulphite. Even with heat treatment to eliminate vegetative cells, confirmation may be required since there are facultative anaerobic sporeformers (e.g., *Bacillus licheniformis*) that can also grow, depending on the medium employed (Fischer *et al.*, 2012).

While SRC are not widely used in foods as a hygiene indicator, a review of its key properties in relation to those expected of an effective indicator organism (Cordier and ICMSF (2013)) shows that SRC would fulfil most of them:

- History of concomitant presence of indicator(s) and associated pathogen or its toxin;
- Presence usually at higher levels than associated pathogen;
- Presence indicative of an increased risk of faulty practices or faulty processes;
- Survival or stability similar to or greater than that of the target pathogen;
- Growth behaviour similar to or greater than that of the target pathogen;
- Easily detectable and/or quantifiable;
- Identifiable characteristics need to be stable;
- Methods for indicator organisms need to fulfil the same requirements as the one for the pathogen, i.e., they need to be reliable and validated; in addition, they should be more rapid and less expensive;
- Quantitative results should show a correlation between indicator concentration and level of the pathogen;
- Results need to be applicable to process control;
- Analyst health is not at risk;

Testing for SCR is therefore deemed an appropriate indicator of process hygiene with respect to the control of anaerobic sporeformers. For illustration, limits for SRC currently used in trade range from a regulatory limit in the Russian Federation of 25 – 100 cfu/g for import of dairy derivatives, including milk proteins (Russian Federation, 2008) and advisory maximum levels of 10-25 cfu/g in the USA (US Dairy Export Council, 2013).

**Use of tightened sampling plans following loss of process control**

There appear to be no standardized sampling plans for the analysis of foods, with the exception of bottled natural mineral waters (CAC, 1985). However, based on the ICMSF cases approach, this would likely be considered as a Case 5 sampling plan, i.e., indicator organism with no potential for growth under the normal handling conditions. Table 2 compares two different microbiological limits for SRC and shows the relationship between the number of samples analyzed via a 2-class attribute plan, using a plating method, and the mean Log(CFU/g) that would just be rejecting the lot as non-compliant, assuming a high degree of confidence (e.g. 95%) in the rejection.
Table 2: Relationship between the numbers of samples analysed and the mean Log (CFU/g SRC) just achieving 95% confidence of rejecting non-compliant lots using either a microbiological limit of 10 or 100 CFU/g.

<table>
<thead>
<tr>
<th>Number of samples analyzed</th>
<th>Mean Log(CFU/g) that would just achieve 95% confidence of rejecting non-compliant result</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Limit = 10 CFU/g</td>
</tr>
<tr>
<td>1</td>
<td>0.42</td>
</tr>
<tr>
<td>3</td>
<td>-0.63</td>
</tr>
<tr>
<td>5</td>
<td>-1.00</td>
</tr>
<tr>
<td>10</td>
<td>-1.42</td>
</tr>
<tr>
<td>15</td>
<td>-1.63</td>
</tr>
<tr>
<td>30</td>
<td>-1.95</td>
</tr>
</tbody>
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These calculations assume that 1.0 ml of a $10^{-1}$ dilution of each food sample is plated on an individual plate. Thus each sample contains 0.1g of the original food. For the purposes of this example, a log normal distribution with a standard deviation of 0.8 was assumed, and that all samples were randomly and independently taken. If there were concerns about the distribution of the samples within a lot not being randomly distributed, then one would need to move toward the larger number of randomly selected samples or alternate sampling plans such as a random stratified sampling regime.

If a production lot exceeds the established limit selected, increased sampling of batches is advisable. The level of increased sampling is a management decision but could consider, for example, an increase in sampling by a factor of 5 – 10 until SRC values return to the normal baseline for the product and/or the source of the contamination has been determined and rectified. Control chart plotting of SRC may permit corrective actions to be taken before the rejection criterion is exceeded (ICMSF, 2002).

Recommendations

Given that *Clostridium botulinum* is not considered a hazard in infant formula (CAC, 2008), the important constraints related with the analysis of *C. botulinum* likely being heterogeneously distributed at very low levels normally in powdered dairy products, the ICMSF does not recommend routine testing for this pathogen. End-product testing should be limited to samples investigated during an outbreak to determine its source, thus allowing regulatory authorities to take appropriate measures to protect consumers, such as the recall of the incriminated product.

It is recognized that there is no direct mathematical correlation between levels of spores of sulphite-reducing clostridia (SRC) and those of *C. botulinum*. The ICMSF nevertheless concludes that testing for SRC is appropriate as an indicator of process hygiene with respect to the control of anaerobic sporeformers. Therefore, the level of SRC can be used to determine adherence to Good Hygiene and Manufacturing Practices during the production of dehydrated dairy ingredients or products such as powdered infant formulae. SRC levels exceeding a limit of m = 100 cfu/g, for example, would point to conditions potentially conducive to the multiplication of anaerobic clostridia in the processing lines, or some source of external contamination. Considering the characteristics and behaviour of various types of clostridia, such levels would then also point to conditions possibly supporting multiplication of *C. botulinum*, when present, beyond normal low levels. In contrast, SRC levels below this limit would confirm the effectiveness of the established hygiene control measures, and hence a negligible risk for *C. botulinum*.
Lots within the established limit could be used as intended. If a lot exceeded this limit, an investigation into possible causes would be warranted together with actions to prevent further occurrence of unacceptable levels. With regard to the affected lot, this should be subject to appropriate actions to mitigate the potential risk. Such actions could range from the rejection of the lot in question, its use in products targeted towards non-sensitive consumers or for products manufactured with a kill-step designed to eliminate clostridial spores.

**Bibliography**


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