SUMMARY OF LA LETTRE DE CECALAIT, N° 36 (1st quarter 2001)

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Results of the European Programme on *Clostridium perfringens*

Before accepting a standard as an European standard, CEN (*the EU standardization body*) requires precision data, which must have been validated by collaborative studies, as specified in standard ISO 5725. At the end of 1996, the European Community launched a 4 year project to validate six ISO microbiological methods for acceptance as standards. These are the methods of detection and/or enumeration of the following pathogens : *Bacillus cereus, Listeria monocytogenes*, coagulase positive *Staphylococcus, Clostridium perfringens*, *Salmonella.*

We have already reported on *Bacillus cereus* (see La Lettre de CECALAIT n° 26), on *Listeria monocytogenes* (see La Lettre de CECALAIT n° 30) and on coagulase positive staphylococci (see La Lettre de CECALAIT n° 35). Since then, the study on *Clostridium perfringens* is also finished. Its results and conclusions were shown during a meeting among the contractors in december 2000 and the final report will soon be issued.

Three contractors are involved in this project :

- AFSSA, France, also coordinator of the project,
- RIVM, Netherlands,
- MAFF-CSL, United Kingdom.

Each of them is in turn responsible for a study. RIVM was thus leader for the study on *C. perfringens*.

Among sub-contractors, CECALAIT was responsible for preparation, development, definition of preservation parameters and shipping of the cheese samples.

1) *CLOSTRIDIUM PERFRINGENS :* GENERAL POINTS AND ENUMERATION METHODS

It is an anaerobic, Gram positive, sporeforming rod, able to produce several food poisoning toxins during sporulation. It is considered as the 3rd cause of foodborne illnesses throughout the world (in THOLOZAN et al.).

It is widely distributed in the environment and frequently occurs in the intestines of humans and healthy animals. It often contaminates meat based food, either directly, or via the environment. Spores persist in soil, sediments and areas subject to fecal pollution. In favourable conditions : temperature between 15°C and 50°C, pH about 7, aw between 0.93 and 0.945, its generation time is very short. In the cold, some strains may still grow, but usually growing stops quickly : the number of vegetative cells is reduced, but spores survive. On the contrary, most spores are killled during cooking, but in some strains, they resist, allowing the microorganisms to multiply in prepared food, especially when it is cooled down too slowly.

Perfringens poisoning is caused by the consumption of food contaminated by quite a large number of vegetative cells (from 10^5 to 10^8/g of food, according to the litterature!) : the cells multiply in the intestine and sporulate, releasing toxins. Symptoms : diarrhea and intense abdominal cramps, begin 6 to 48h after ingestion of contaminated food and are usually over within 24h. Most outbreaks of perfringens poisoning implicated meat, meat products or gravy.

↳ REGULATIONS

Microbiological criteria for food seldom refer to *C. perfringens* itself, at least in the french regulations. For most foods (meat, pastries…), neighbouring criteria are specified, wider and not so well defined, eg sulfite-reducing *Clostridia* or sulfite-reducing anaerobes. In dairy products, these criteria only concern some milk-based products intended for particular nutritional use. When these products « were not heated in their bottles and might need a liquid adjunction before consumption », the criteria are :

In 1g of dry product or 10 g of liquid product :

- *C. perfringens* : absence,
- sulfite-reducing *Clostridia* at 46°C : < 10.

(French regulations : ministerial order of 1978/3/30, in note de service DGAl n° 2000-8155)

↳ METHODS

➔ ISO 7937 (1997) and EN 13401 (1999)

ISO 7937 (1997) is the horizontal reference method for the enumeration of *Clostridium perfringens.* CEN standard EN 13401 (1999) is practically the same, except for confirmation of presumptive *C. perfringens*. Indeed, it allows a choice between confirmation using lactose-sulfite medium (as in ISO 7937 (1997)) or using motily-nitrate & lactose-gelatine medium (as in ISO 7937 (1985)).

So both techniques have been included in this validation study in order to compare their performances and later to possibly harmonise the two standards.

Principle of standards ISO 7932 and EN 13401 is as follows, after preparation of the initial suspension.

- inoculate two sterile empty Petri dishes with the initial suspension or each of the serial dilutions,
- Pour egg-yolk free tryptose-sulfite-cycloserine agar (SC), maintained at 47°C and mix well,
- after solidification, add an overlayer of the same SC agar,
- incubate anaerobically at 37°C for 20h,
- count the black colonies of presumptive *C. perfringens*,
- confirm characteristic colonies (usually 5), retained for the enumeration.

➢ In standards ISO 7937 and EN 13401, the confirmation method is based on high gas production and the presence of a black precipitate, in lactose-sulfite medium after anaerobic incubation at 46°C.

➢ However standard EN 13401 describes an alternative confirmation method using the combination of two other tests which must be performed with the **same** well-separated characteristic colony :

- the first test uses the motility-nitrate reduction medium, where, after anaerobic incubation at 37°C, *C. perfringen*s are non motile and reduce nitrate to nitrite, thus forming a strong red colour after adding a nitrite-detection reagent,
- The second test is based on high gas production and the presence of a yellow colour in lactose-gelatine medium, then on gelatine liquefaction.

The study was aimed at the determination of repeatability, r, and reproducibility, R, values for the method decribed in ISO 7937, but with each of the confirmation techniques.

2) COLLABORATIVE STUDY

As in the other studies of the project, the samples used were :

- reference material (capsules prepared by RIVM containing milk powder contaminated with spores of *Clostridium perfringens*),
- three different artificially contaminated food matrices :
  - raw milk cheese,
  - dried meat, prepared by MAFF-CSL,
  - dried animal feed, prepared by RIVM. But, due to the stickiness of this matrix, direct contamination was impossible. Therefore artificially contaminated milk powder was added to the feed.

They were all inoculated, at different inoculum levels, with spores of an appropriate *C. perfringens* strain*, and also with a simulated autochthonous flora for cheese and meat. For the feed however, the natural contamination flora was kept as background flora.

* originated from food or from patient material.

The final contamination levels are given in table 1, page 3 in La Lettre de CECALAIT n° 36.

Homogeneity and stability were checked before the beginning of the study.

The collaborative study took place in january and february 2000 and involved 17 laboratories from 13 European countries.

The analyses were made in blind duplicate and most laboratories tested all samples and performed both confirmation techniques.

3) RESULTS

Shipping and reception of the samples were generally found satisfactory.

Concerning the operating procedure, the incubation conditions in the lactose-sulfite confirmation medium varied between participating laboratories. Indeed the procedure described in ISO 7937 did not seem clear enough. Thus some laboratories incubated test tubes anaerobically, whereas others did not. Furthermore, these had difficulties with reading of the gas formation. However, these deviations were regarded as of minimal influence on the final results and did not lead to any exclusion of results.

After log transformation and exclusion of outliers, using Duncan's Multiple Range test, repeatability and reproducibility were determined. As usual, for the calculation ISO 5725 was followed, but also standard project EN ISO 16140 (using the median value) which seems to fit better to microbiological methods.

Tables 2 and 3 in La Lettre de CECALAIT, pages 3 and 4 show the results obtained with the latter method. Nevertheless, the values obtained, using either method of calculation were almost the same.

Tables 2 and 3 show that repeatability and reproducibility vary somewhat among the different food types and the different contamination levels. As expected, the lower values were obtained with reference material. The higher ones were obtained for the dried animal feed at the lowest contamination level. These variations might be explained by the difference in the matrix, or by the different ways of samples contamination. Most of the time, the highest precision values were observed for the lowest contamination level. This was the case for cheese and feed, but was not so clear for meat.

However it was possible to calculate the average repeatability and reproducibility values as the arithmetic mean of the values obtained in the three levels (see table 4, page 4 in La Lettre de CECALAIT).

As in tables 2 and 3, table 4 also shows that the precision data obtained with either confirmation technique are very similar. As both techniques show equal performance, it seems desirable to leave the user the choice which one to perform.

4) CONCLUSION

The conclusions of this study of the reference methods for the enumeration of *Clostridium perfringens* found both ISO 7937 and EN 13401 methods satisfactory. Nevertheless the following recommendations to CEN and ISO were drawn up and will be presented at the next meeting for this programme in June 2001, in Bern :

- to include in both standards the precision data calculated in this study using project EN ISO 16140.
- to allow a choice in ISO 7937 between two techniques for confirmation of presumptive *C. perfringens* colonies : one, using lactose-sulfite medium, the other the combination of motility-nitrate and lactose-gelatine medium. This will lead to the harmonisation of standards ISO 7937 and EN 13401.

◆ to improve, in both texts, the description of the incubation conditions for lactose-sulfite medium confirmation test. It will be necessary to specify if anaerobic conditions are compulsory or optional.

The list of abbreviations and bibliographic references are in « La Lettre de CECALAIT »