SUMMARY OF « LA LETTRE DE CECALAIT », N°30 (JULY 1999)

METHODS OF ANALYSIS OF LISTERIA MONOCYTOGENES
(Abstract of the lecture given by Mrs Rollier at CECALAIT's Annual General Meeting)

Since the beginning of 1999, public attention in France has been focused on safety problems in food associated to *Listeria monocytogenes*.

Listeriosis is a rather rare disease (225 cases in France in 1997), mostly associated to food. Most healthy people show no or influenza-like symptoms without any consequence. But, for target people, complications may be severe, usually meningitis or septicemia, which are often fatal. The main target populations are elderly people, new-borns, immunocompromised persons, pregnant women. Infections during pregnancy may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. Listeriosis is caused by *L. monocytogenes*, of which the infective dose is unknown but is believed to vary with the strain, susceptibility of the victim and contamination conditions. This bacterium is ubiquitous and very hardy.

In any case, dairy regulation, eg EC directive 92/46 specifies the absence of *Listeria monocytogenes* usually in 25 g of product (cheese, milk…). So the detection of this bacterium is of great importance in food hygiene and faster, easier and more specific methods are expected.

UBIQUITOUS AND VERY HARDY BACTERIUM

It is a Gram-positive bacterium, motile at 22°C, but not at 37°C, aero-anaerobic. It is quite hardy and resists remarkably well to chemicals and severe temperature, pH or salinity conditions as is shown in table 1 in La Lettre de CECALAIT.

Therefore, it can been found everywhere : air, soil, plants, food, healthy carriers….In food, incidence data collected France showed (1993 to 1995 data) a rather high incidence in meat products, then decreasing from seafood to vegetables, pastries and dairy products. However, in these latter products, contamination is often specific to soft cheeses, more heavily contaminated than other foods.

The epidemiologic survey since the 80s shows that *L. monocytogenes* has been associated to very different kinds of foods, including cabbage, raw-meat sausages, raw and smoked fish, and dairy products such as soft cheeses, pasteurized milk…It also shows that serotype 4b is usually responsible. In the last years, the duration and the number of cases in epidemics has tended to decrease. This is certainly linked to the more frequent official and self controls during food processing and marketing and to the improvement in methods of analysis.

EVOLUTION OF THE METHODS

It was necessary to evaluate their evolution as the classical methods are very complex and time consuming. Table 2 in La Lettre de CECALAIT shows the evolution in the methods of detection and enumeration of *L. monocytogenes*.

Reference methods, such as ISO 11290-1 and –2 (1997 and 1998) and IDF 143A :1995 also improved, as is shown in table 2. But, they still remain much too time consuming for routine purposes in dairy laboratories. So a lot of alternative, quick methods appeared as shown in table 3 in La Lettre de CECALAIT. Most of them are validated (AFNOR, AOAC). However, as the enrichment steps still remains necessary, it is difficult to take less than 48h.

As CECALAIT has been organizing ringtests for Listeria detection since 1993, we could observe how laboratories followed the evolution of methods. Figure 1 in La Lettre de CECALAIT thus compares enrichment,isolation media and identification techniques used in the first ringtest (March 1993) and in the latter one (April 1999).

However, even quick method users often use reference methods for confirmation. So the conclusions of the European Project of validation of ISO 11290-1 & -2 methods of detection and enumeration of *L. monocytogenes* are particularly interesting.

EUROPEAN PROJECT OF VALIDATION OF ISO MICROBIOLOGICAL METHODS

This European Community project was elaborated to validate six ISO microbiological methods for acceptance as CEN standards. It aims at determining, by collaborative studies, the precision data or performance characteristics of detection and/or enumeration methods of following pathogens : *Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Salmonella.*

We have already reported on *Bacillus cereus* (see La Lettre de CECALAIT n° 26). Now the studies about *Listeria monocytogenes* are also finished.

Three contractors are involved in this project :

- AFSSA in France, also coordinator of the project,
- RIVM in the Netherlands,
- MAFF-CSL in the United Kingdom, « trial leader » for the validation of the methods concerning *L. monocytogenes.*

Among sub-contractors are :

- Institut Pasteur in Lille, for checking of the experimental protocols,
- ENILBIO in Poligny for statistical treatment of the detection method,
- CECALAIT for preparation, developement, definition of preservation parameters and shipping of the cheese samples.

Each validation study uses reference material (capsules prepared by RIVM) and three different artificially contaminated food matrices : cheese, dried meat, prepared by MAFF-CSL, another dried food. This study concerned egg, prepared by RIVM.

They were inoculated, at different inoculum levels, with a natural or simulated autochthonous flora, with *L. monocytogenes* and *L. innocua*, isolated from food.

The final contamination levels are given in table 4 (two parts) in La Lettre de CECALAIT.

The final participation in the collaborative studies involved :

- 19 laboratories from 14 European countries for the detection method, each analysing 15 samples of each food matrix (5N, 5B, 5H) and 5 reference capsules
- 21 laboratories from 16 European countries for the enumeration method, each analysing 8 samples of each food matrix (2N, 2B, 2M, 2H) and 2 reference capsules.

The ISO methods used necessitate several successive stages after preparation of the initial suspension :

- for ISO 11290-1, detection
  - primary enrichment in Fraser½ broth
  - secondary enrichment in Fraser broth,
  - plating out on OXFORD and PALCAM agars from cultures obtained after each of the two enrichment stage

• confirmation of genus, then of species by appropriate morphological, physiological and biochemical tests (catalase reaction, Gram staining, motility, optional Henry illumination; haemolysis, carbohydrate utilisation and CAMP test). *NB : in the collaborative study, only catalase, carbohydrate and CAMP tests were compulsory*

◆ for ISO 11290-2, enumeration

• resuscitation for 1h at 20°C,
• surface plating on PALCAM agar of the sample itself, if liquid, or of the initial suspension (prepared in either buffered peptone water or base of Fraser 1/2 broth as diluents)
• confirmation of genus, then of species as above
• from the number of confirmed colonies, calculation of the number of *L. monocytogenes* per g of test sample.

↳ Results

➢ Detection

They are generally satisfactory : overall sensitivity *ca* 86%. There is no evidence of difference between performance of OXFORD and PALCAM agars. They confirm that a dual enrichment has to be maintained.

➢ Enumeration

After log transformation and exclusion of outliers, repeatability and reproducibility were determined.

These values are given in table 5 in La Lettre de CECALAIT.

r : repeatability. In a log scale, it means that the log difference between two replicates performed in the same laboratory has a 95% probability of being lower than r

R : reproducibility. In a log scale, it means that the log difference between the same analyses performed in different laboratories has a 95% probability of being lower than R

There are great differences between the different food matrices, which shows the difficulties of the method, considering the poor selectivity of the agar for *L. monocytogenes.*

↳ CONCLUSIONS

The conclusions of the European Project give the following recommendations to CEN and ISO :

➢ for ISO 12290-1

◆ to incorporate in the standard the performance characteristics derived from the study : sensitivity, specificity, accordance, concordance, odds ratio ✸

✸ sensitivity : % of samples that are correctly found to be positive
specificity : % of samples that are correctly found to be negative
accordance : something equivalent to repeatability in quantitative studies
concordance : something equivalent to reproducibility in quantitative studies
odds ratio : parameter to assess the degree of inter-laboratory variation, by comparison between the magnitudes of accordance and concordance.
More precise definitions available from CECALAIT on request

➢ for ISO 12290-2

◆ to incorporate in the standard all precision data derived from this study as it is impossible to find out a mean value considering the dispersed results

➢ for ISO 12290-2

◆ to include the correct value (2%) of the sheep erythrocytes used in the microwell haemolysis test.

◆ to launch studies in order to find a medium allowing a better differenciation of *L. monocytogenes* from other species, especially *L. innocua*.

GENERAL CONCLUSION

The conclusion of this European project will lead to a revision of ISO 11290-1 and –2 standards.

Furthermore, with the development of faster and more specific methods, the detection of *L. monocytogenes* should become easier and faster. So the prevention of listeriosis should become easier too. However, as this bacterium is ubiquitous and hardy, it should be considered that « 0 risk is impossible » and appropriate public information should be developed, especially towards susceptible populations

Bibliographic references are in « La Lettre de CECALAIT »

INTERESTING NEW STANDARDS

↳ INTERNATIONAL DRAFT STANDARDS

ISO 7218/A1. MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS General rules for microbiological examinations

ISO 6888-1 & -2 MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS. Horizontal method for the enumeration of presumptive *Escherichia coli*.
Part 1 : colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indolyl-β-D glucoronic acid
Part 2 : colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-β-D glucoronic acid

➢ **AOAC International** published the 1999 update of the Official Methods of Analysis, 16th edition (OMA)

↳ Some of you asked us for details about **ISO 12081 December 1998,** titrimetric method of determination of calcium content in milk (see La Lettre de CECALAIT, n°29). Actually, it is equivalent to IDF standard 36A :1992, where calcium is precipitated as calcium oxalate and the precipitate then titrated with potassium permanganate

INTERESTING RECENT EEC REGULATION

As usual, **regulation n° 2377/90** of the Council concerning maximum residue limits of veterinary drugs in foods of animal origin has been amended.

• the regulation itself was amended by **regulation 1308/1999** of 1999/6/15 (JOCE L156 on 1999/6/23) (mostly about European Drug Evaluation Agency)

• annex I was amended by **regulations 997/1999** and **998/1999** of 1999/5/11 (JOCE L 122 on 1999/5/12),

• annex II was amended by **regulations 953/1999** of 1999/5/5 (JOCE L 118 on 1999/5/6)**, 997 and 998/1999,**

• annex III was amended by **regulations 953/1999, 954/1999** of 1999/5/5 (JOCE L118 on 1999/5/6), **997/1999.**

New residue limits were then inserted in the tables.

Commission Decision 1999/309/CE of 1999/4/23 on the 1999 work programme relating to the protein content of the main milk products (JOCE L119 on 1999/5/7).

Commission Regulation (EC) No 881/1999 of 1999/4/29 amending Regulation (EC) No 1854/96 establishing a list of reference methods to be applied for the analysis and quality evaluation of milk and milk products under the common market organization (JOCE L111 on 1999/4/29).

Commission Directive 1999/39/EC of 6 May 1999 amending Directive 96/5/EC on processed cereal-based foods and baby foods for infants and young children (JOCE L124 on 1999/15/18).
&
Commission Directive 1999/50/EC of 1999/5/25 amending Directive 91/321/EEC on infant formulae and follow-on formulae (JOCE L139 on 1999/6/2)

Both texts are about the maximal residue limits of pesticids in these foods (0.01 mg/kg of food)

Official Journals of the European Communities of the last 45 days may be consulted on Internet http://europa.eu.int/eur-lex
Older texts may be ordered on Internet http://www.eudor.com

FORTHCOMING EVENTS

➲ REMINDER OF IDF EVENTS

➢ **15 – 18th September 1999** : ATHENS (Greece) 83rd annual sessions

For information, please contact
IDF
Secretariat
41, square Vergote
B-1030 BRUXELLES
BELGIUM
Fax : 32/.2.733.04.13
e-mail : info@fil-idf.org
http://www.fil-idf.org

➢ **4 – 7th October 1999** : SAN FRANCISCO (USA), nutrition week

For information, please contact
IDF or
Anne Divjak, secretary USNAC
1250 H Street NW, Suite 900
WASHINGTON DC 20005
USA
tel : 1/202.737.4332
fax : 1/202.331.7820
e-mail : adivjak@idfa.org

➲ REMINDER

➢ **1 – 4th August 1999** : IAMFES ANNUAL MEETING, in DEARBORN (Michigan, USA)

IAMFES : International Association of Milk, Food and Environmental Sanitarians
For information, please contact

IAMFES
6200 Aurora Ave
Suite 200W
DES MOINES
IA 50322-2863
ETATS-UNIS
Tel : +1/800.369.6337 ou +1/515.276.3344
fax : +1/515.276.8655
e-mail : iamfes@iamfes.org

http://www.iamfes.org

➢ **26 – 30th September 1999** : HOUSTON, (Texas, USA), 113th AOAC International annual meeting

for information, please contact **IDF**

➢ **3 – 10th October 1999** : SYDNEY (Australia), 10th World Congress of Food Science and Technology

For information, please contact

IUFoST Congress 10, GPO Box 128,
Sydney
NSW 2001
AUSTRALIA
Tel : +61 2 9262 2277
Fax : +61 2 9262 2323
e-mail : Tourhosts@tourhosts.com.au

➢ **18 – 21st October 1999** : METROLOGIE 99, 9th International Congress of metrology in BORDEAUX (France)

For information, please contact

Secrétariat Général Métrologie 99
Sandrine GAZAL
Maison de l'entreprise
429, rue de l'Industrie
34966 MONTPELLIER CEDEX 2
Fax : +33/4.67.91.33.43

➲ OTHER IDF EVENTS

➢ **17 – 19th August 1999 in** MUTARE (Zimbabwe)

Quality management for small and medium-sized dairy processors

For information, please contact **IDF** or

IDF Zimbabwe National Committee
Mrs P. BORLAND
National Secretary of IDFZ
Zimbabwe Dairy Services Association
PO Box CY 2026 Causeway, HARARE
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tel : 263/4.729177
fax : 263/4.704545
e-mail : cuthbert@primenet.co.zw

➢ **14th September 1999** in ATHENS (Greece)

Workshop on organic milk

➢ **7 – 8 th November 1999** in FRANKFURT, (Germany)

Symposium on overcoming barriers to international trade of food and dairy products

For information, please contact **IDF**

➢ **13 – 16th March 2000** in BANFF (Canada)
Cheese ripening and technology

For information : **IDF** or
Dr P. Jelen
Dpt Agricultural, Food and Nutritional Science
University of Alberta 2-06 Agfor Centre
EDMONTON AB T6G 2P5
CANADA
tel : 1/403.492 2480
fax : 1/403.492 8914
e-mail : pjelen@afns.ualberta.ca

➲ OTHER EVENTS

➢ 5 – 6th October 1999 in JOUY EN JOSAS (France)

International symposium on PLS methods

For information please contact

CIRIA – CERESIA Symposium PLS
Concesa Olivier
1, avenue Herbillon,
94160 SAINT MANDE
tel : 01.43.74.95.20
fax : 01.43.74.17.29
e-mail : ciria@calva.net

DETERMINATION OF MILK PROTEIN

PART 2 : EVALUATION OF THE CECIL 2000 SPECTROPHOTOMETER

In France, the official method for protein determination, for milk payment purposes is the Amido Black dye-binding method. Many dairy laboratories also use it, either directly, or as as secondary reference method for the calibration of infrared instruments.

A spectrophotometer is necessary to perform the method. Until now, French laboratories have mainly been using three types Vital 33, Promilk and Astor. As the first two are no longer manufactured, new ones have been developped and marketed. In view of the importance of the Amido Black method as a secondary reference method for the calibration of infra red instruments, French authorities decided that these new spectrophotometers had to be subjected to a national procedure of approval. This requires evaluation tests by an expert laboratory. CECALAIT has therefore recently evaluated the analytical characteristics of two new spectrophotometers ATL 33 (distributed by Humeau) and CECIL 2000 (distributed by Grosseron).

In the last issue of « La Lettre de CECALAIT », we reported the evaluation of the ATL 33. In this issue, we are dealing with CECIL 2000.

APPARATUS

CECIL is a spectrophotometer, equiped with a holographic network that allows it to operate within the wavelength range of 325 to 1000 nm, but the usual wavelength chosen for the method is 620 nm. It is composed of a single item, equipped with an integrated printer, and also a screen and a keyboard in order to run the apparatus.

TESTS PERFORMED

Tests were performed in November 1998, using the following procedure :

- milk volume : 0.8 ml
- addition of 16 ml of Amido Black solution
- swirling for 10 mn
- centrifugation (350g) for 5 mn.
- measuring of the absorbance of the supernatant at 620 nm.

The following characteristics were evaluated, according to IDF standards 128(1985) and 135B(1991):

- stability
- carry-over effect
- linearity
- repeatability
- accuracy

❶ **Stability** was evaluated by analysing a set of four amido-black solutions diluted at 4 distinct levels, every 15 mn for half a day. The results show a standard deviation of reproducibility of : **0.00 to 0.02 g/kg or 0.00 to 0.09%,** which corresponds to a good stability.

❷ CARRY-OVER EFFECT

This was evaluated by analysing amido black solutions and water, 10 times, in the following sequence : water-solution 1-solution2, at three different concentrations.

The carry-over effect (Tc %) was estimated with following equation :

$$Tc\ \% = [\Sigma(\text{SOLUTION 2}) - \Sigma(\text{SOLUTION 1}) / \Sigma(\text{SOLUTION 2})] \times 100$$

Tc values are in the interval of **0.0 to 0.4%.**

These values comply with the maximum limit of 1% allowed in routine methods of determination of milk composition, used for milk payment purposes.

❸ LINEARITY

Linearity was evaluated by analysing :

- a set of diluted amido black solutions within the usual absorbance range.
- a set of milk samples, with protein content ranging from 20 to 45 g NP/kg

Linearity was estimated by using simple linear regression. The results obtained on the set of milk samples are shown in Figure 2, in « La Lettre de CECALAIT ».

The spectrophotometer is linear in the range 25 to 36 g NP/kg. This result was obtained using the curvilinear calibration method, given in the configuration of the CECIL.

❹ REPEATABILITY

Repeatability was evaluated by duplicate analysis of 100 individual milk samples (preserved with 0.02% bronopol).The results are given in table 6 in « La Lettre de CECALAIT ».

These values are very close to those obtained with the former spectrophotometers, such as VITAL 33.

❺ ACCURACY

Accuracy was evaluated by duplicate analysis of 100 individual milk samples, using CECIL 2000 and VITAL 33 (CECALAIT's instrument), in parallel. The instruments were calibrated by using CECALAIT commercial Amido Black SMRs at three concentrations : 25, 30 and 26 g NP/kg.

Accuracy was estimated by using the residual standard deviation of the regression where Y represents the values given by VITAL 33 and X the values given by CECIL. Figure 3 in « La Lettre de CECALAIT » shows the results in the 25 to 36 g NP/kg range.

- **The mean bias, not significant, is 0.00,**
- **the regression slope is significantly different to 1**

because the relationship between the two instruments is not fully linear. The deviations are mostly seen for the extreme values of the NP range and are however very low

- **the residual standard deviation of the regression is 0.14**.

This shows a good agreement between the results given by the two instruments, especially in the 25 to 36 g NP/kg range.

➢ **In conclusion**, the analytical characteristics of CECIL 2000 have been found satisfactory. Therefore, it received an official national approval for protein determination for milk payment purposes.

abbreviations : MAP (Fr) = NP = protein nitrogen
SMR : secondary reference material

Bibliographic references are in « La Lettre de CECALAIT »

LIST OF BIBLIOGRAPHIC REFERENCES

It is the list of references that we noticed in our litterature survey during the past months and that we decided to put into our data base on dairy analytical techniques. Should you be interested in any of these references, contact us, please.

NB : we remind you that we can copy neither book nor standard.