SUMMARY OF LA LETTRE DE CECALAIT, N° 38 (3rd quarter 2001)

(Translation : A. BAPTISTE, Correction : H. LAMPRELL)

Evaluation of ISO 6579 method for the detection of *Salmonella spp.*

(based upon the lecture given by Mrs BOHNERT, AFSSA at CECALAIT's Annual General Meeting 2001)

At the end of 1996, the European Community launched a 4 year project to evaluate the precision of six ISO microbiological methods concerning detection and / or enumeration of pathogens in food. Indeed, before accepting them as standards, CEN urges the need for precision data, which must have been validated by collaborative studies, as specified in standard ISO 5725. This has already been done for *Bacillus cereus, Listeria monocytogenes* (detection and enumeration), coagulase positive *Staphylococcus* and *Clostridium perfringens* (cf Lettres de CECALAIT n° 26, 30, 35 and 36). The validation of the horizontal method for the detection of *Salmonella* spp. was the last step in the project.

Its current version was described in standard ISO 6579, issued in 1993 but under revision since 1997. Therefore, it seemed better to wait and evaluate the method to be used in the years to come. That's why the horizontal method for the detection of *Salmonella* spp. was the last evaluated, using the protocol described in draft EN ISO 6579, issued in 2000.

Earlier validations had involved about 20 European laboratories. But this time, the study also aimed at the AOAC recognizing the ISO method, so that this latter method could be used in food export controls towards the USA, instead of the AOAC method. So, some AOAC International US expert laboratories joined the collaborative study too. However, as these methods had to be compared, the experimental procedure became even heavier.

As before, three contractors were involved in this project :

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

They were each in turn responsible for a validation. Thus, AFSSA (Ploufragan unit) leaded the study concerning *Salmonella* spp. in Europe ; an american laboratory (BioControl Systems) supervised the study in the USA.

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

1) ABOUT *SALMONELLA*

✎ GENERAL INFORMATION

Salmonella are involved in numerous foodborne disease outbreaks (more than 40 000 confirmed cases annually in the U.S). Moreover the incidence of salmonellosis appears to be rising both in the U.S. and in other industrialized nations

There is a widespread occurrence in animals : poultry, swine, rodents, reptiles and cats. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchens. The foods most associated are ground and raw meats, sausages, poultry, eggs and egg products (including salad dressings and cream-filled desserts and toppings), fish and shellfish, milk and dairy products. However, there are always more cases in summer as they grow faster in non refrigerated food.

Salmonella are rod-shaped, motile bacteria (with the exception of the nonmotile *S.* Gallinarum-Pullorum serovar, a bird pathogen), facultative aero-anaerobic, non-sporulated. Their optimal growth temperature is 35/37°C ; but some strains are able to multiply from 5°C to 45/47°C. However, their growth is slowed below 10°C. They are not totally killed by pasteurisation, but not by freezing.

Their optimal growth pH is 6.5 to 7.5, but they usually resist from pH 4.5 to 9.0. Some strains even seem able to survive in a more acid environment. They can also survive for long periods in dry food. Moreover, at least one strain resisting different class antibiotics has been reported.

Salmonella bacteria are all potential pathogens for humans or animals. *Salmonella* Typhi and Paratyphi, even at a low infective dose, produce typhoid or typhoid-like fever in humans (fatality rate : 10%). Other milder forms of salmonellosis generally require a high infective dose. Then bacteria multiply in the intestines which results in the onset of acute symptoms in 6 to 48h : abdominal cramps, frequent, painful and intense vomiting, diarrhea, nausea, headache, fever….usually lasting for 1 to 7 days.. Most persons recover without treatment, however, FDA reports on 2 deaths for 1000 contaminations. People particularly affected are the elderly, infants and immunodepressed patients.

✎ REGULATION

French and European Union regulation require the absence of *Salmonella* in most foods, especially milk, dry milk, cheese, butter, milk based beverages when they are marketed. Directive 92/46* specifies « absence in 25g of raw milk intended for direct human consumption, in 1g of other products. **» French regulation however specifies « **absence in 25g** » for all dairy products (in note de service de la DGAl (n°2686 of 24/10/1996 ***).

** quoted in DG 24, «'overview of microbiological criteria for foodstuffs in community legislation in force », updated june 2001*
*** in directive 94/71 of 13/12/1994 amending directive 92/46*
**** quoted in Note de service n°2001-8090 of 27/06/2001*

2) DETECTION METHODS

➷ EN 12824 OF 1998

It is the **present** horizontal reference method in the European Union for the detection of *Salmonella* spp. It is based upon ISO 6579 :**1993** standard, so it requires the use of :

- selective liquid media : Rappaport-Vassiliadis malachite green magnesium chloride broth and selenite-cystine broth, for the enrichment phase,
- brilliant green phenol red agar (Edel and Kampelmacher medium) and a second free-choice selective plating-out medium, for the isolation phase.

However, compared to ISO standard, it allows 24 additionnal hours for the incubation in Rappaport-Vassiliadis medium.

➷ DRAFT ISO 6579, JUNE 2000

This is a new method which was validated in this study. It is very different from former standard and from EN 12824 standard, particularly on following items :

- the two liquid selective media for enrichment : the first is now Rappaport-Vassiliadis broth containing soya peptone (RVS) ; the second one, selenite-cystine broth, considered as environmently hazardous is replaced by Mueller-Kaufmann tetrathionate-novobiocine broth(MKTTn)
- the two solid selective media for plating out : as before, one is compulsory, the other can be chosen freely. But, the former compulsory brilliant green phenol red agar is now replaced by xylose lysine desoxycholate agar (XLD). Concerning the second medium, it is specified that it should allow the detection of lactose positive *Salmonella*, and also of *S.* Typhi and *S.* Paratyphi.

Nevertheless, biochemical and serological confirmation stages still have unchanged principles, but easier protocols.

The diagram of the procedure described in draft ISO 6579 is in four stages.

- pre-enrichment stage with the sample suspended in buffered peptone water, then incubated 16 to 20 h at 37°C.

- enrichment stage in selective liquid media : with the culture obtained after pre-enrichment stage, inoculation of :

    ➢ 10 ml of RVS broth, with 0.1 ml , then incubation 24h ± 3h at 41.5 ± 1°C
    &
    ➢ 10 ml of MKTTn broth, with 1 ml, then incubation 24h ± 3h at 37°C ± 1°C

    NB 1 : 41.5°C should inhibit competing microflora and still remain below 43°C, where most salmonella may be inhibited
    NB : MKTTn broth should allow the growth of S. Typhi and S. Paratyphi.

- plating out stage on solid selective agar : using each of enrichment cultures, inoculation of :

    ➢ XLD agar
    &
    ➢ another selective plating out medium, allowing the detection of lactose positive *Salmonella*, and also that of *S.*Typhi and *S.* Paratyphi
    then incubation 24h ± 3h at 37°C ± 1°C

- confirmation stage: using one characteristic colony from each selective plating-out medium :

    ➢ biochemical tests on :
    - triple sugar iron agar (TSI)
    - urea broth
    - L-lysine decarboxylation broth
    - β-galactosidase detection broth
    - Voges-Proskauer test broth
    - indole test broth

    &
    ➢ serological tests

    If the first tested colony is negative after biochemical tests, all tests are performed with 4 other characteristic colonies from each plating-out media.

At last, tables giving biochemical and serological reactions of *Salmonella* allow these colonies to be classified as *Salmonella* or not as *Salmonella*.

This draft standard was examined again during the ISO standing Comittee SC9 meeting in June 2001 in Bern. Some points still remain controversial, especially :

- replacement of selenite-cystine enrichment broth by MKTTn and compulsory use of XLD agar (IDF, for instance, disagrees),
- should the confirmation stage stop after biochemical tests without or almost without serotyping?
- is O antigen confirmation adapted for all countries ?

Moreover, the composition of some media yet seems rather ill defined. For instance, there are 4 different commercial compositions of MKTTn broth. Preparing RVS broth is not easy either, especially for the magnesium chloride solution : the concentration specified in the litterature does not seem to be met by all commercial media.

➷ AOAC METHOD

AOACI OMA 995.20 method for the detection of *Salmonella* (1999 final action) applies to highly contaminated foods and poultry feed. Its protocol was revised in order to include low microbial load foods, ending in AOACI OMA 2000.06 method (submitted to Microbiology Committee). Both methods were used here.

Their diagram of procedure is also in four stages.

- pre-enrichment stage with the sample suspended in lactose broth (for these food types), then incubation 24h ± 2h at 35°C ± 2°C.

- enrichment stage in liquid selective media : with the culture obtained after pre-enrichment stage, inoculation of :

➢ Rappaport-Vassiliadis broth (RV), with 0,1 ml, then incubation 24h ± 2h at 42°C ± 0.2°C

&

➢ tetrathionate broth, with 1 ml.

Here, incubation differs according to the microbial load of foods:

- for low microbial load, incubation 24h ± 2h at 35°C ± 2°C,
- for high microbial load (e.g. poultry samples in this study), incubation 24h ± 2h at 43°C ± 0.2°C ,

• isolation stage : all enrichment tubes are streaked on selective agar plates :

➢ XLD agar and Hektoen enteric agar (HE), both incubated 24h ± 2h at 35°C ± 2°C

&

➢ bismuth sulfite agar(BSA), incubated 24h ± 2h, examined, then incubated for a further 24h at the same temperature.

• confirmation stage : using two characteristic colonies from each plate, two steps tests :

➢ first step : stabbing and streaking on TSI and lysine iron agar (LIA) slants, incubation 24h ± 2h at 35°C ± 2°C

➢ second step : using presumptive positive colonies from each of these slants, confirmation by the urea test, other biochemical tests, then serological tests.

NB : even when the tests are the same as in the ISO method, media may differ.

As in the ISO method, tables allow classification of the colonies.

3) 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 *Salmonella* Typhimurium),
- three different artificially contaminated food matrices :
  - fresh cheese, prepared by CECALAIT,
  - diced poultry, prepared by MAFF-CSL,
  - egg powder, prepared by RIVM.

They were all artificially inoculated, at three inoculum levels, with appropriate unstressed *Salmonella* strains, originated from food and also with a simulated autochthonous flora of lactics and Gram negative flora for cheese ; Gram positive for meat. Egg powder was contaminated with capsules as well for *Salmonella* as for the Gram positive competing microflora

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

Strains used for poultry and egg powder were respectively *Salmonella* Typhimurium and *S.* Panama*.* Cheese samples were contaminated with *Salmonella enterica*, subsp. *enterica*, serovar Montevideo (also called *S. montevideo*), a lactose positive strain, isolated in the USA, from dried soup. The final contamination levels of cheese samples are given in table 1, page 4 in La Lettre de CECALAIT n° 38.

A pre-trial among the three partner laboratories to check the experimental protocol took place in late 1999. Afterwards, laboratories who wished to, could perform a training exercise with *Salmonella* reference capsules.

The collaborative study finally took place in March and May 2000 and involved 17 laboratories from 12 European countries and 10 US labs.

With 3 matrices, 3 levels and 2 methods, the work load was quite heavy. Therefore protocols of each method were eased in the confirmation stage. Thus, the number of colonies to pick up and of biochemical tests to be performed were decreased. This manner allows no comparison of media : only the whole AOAC and ISO methods can be compared later.

European laboratories could choose their matrices, but had to use ISO method. They could also use the AOAC method.

The analyses were made in blind with 5 repetitions at each level. The results of laboratories, which obtained false positives or with bad results for reference samples (*ie* detection of less than 4 positive samples on 5) were discarded. At last, the number of laboratories, whose results were considered are given in table 2, page 4 of la Lettre de Cecalait n° 38.

4) RESULTS OF THE COLLABORATIVE STUDY

Stability and homogeneity of the samples were found satisfactory, so were, generally, their shipping and reception.

Concerning the operating procedure, there was a great variety in media chosen as free-choice second selective plating-out media :

- 9 labs used BSA (3 different brands),
- 4 labs used BGA (2 different brands),
- 2 labs used Rambach agar,
- MLCB (mannitol lysine cristal violet brilliant green) and HE agar were each used by 1 lab.

Tables 3 and 4 in La Lettre de CECALAIT, pages 5 and 6 show the « precision » results obtained with ISO 6579 draft and AOACI methods. Both are qualitative methods aimed at detecting microorganisms, not at enumerating them. So, usual precision criteria such as repeatability or reproducibility cannot be used here. They were replaced by the criteria developed in the course of the validation of the ISO 11290-1 method for the detection of *Listeria monocytogenes* (in the same European Community project, cf Lettre de CECALAIT, n°30, july 1999) : sensitivity, specificity, accordance, concordance, odds ratio.

Tables 3 et 4 show that performance of the methods vary somewhat among the different food types and the different contamination levels. The ISO method seems to fit particularly well low contamination levels in poultry and egg powder, but is also satisfactory in cheese. AOACI method seems to fit particularly well egg powder and is very satisfactory in cheese. Troubles appeared however for the detection by the AOACI method of low contamination levels in poultry. They were no

longer observed in a further collaborative study, included in the same ISO versus AOACI methods study.

5) FURTHER STUDIES

As AOACI required data concerning the performance of both methods for very low levels of contamination and specificity data for the ISO method in order to recognize this latter method, further studies were necessary. However they did not involve all the participants of the previous study.

➢FURTHER DETECTION STUDIES BY ISO AND AOAC METHODS

AOAC International generally performs analyses at very low contamination levels, where some 25g test portions may be negative. New poultry and egg powder samples were therefore necessary. For cheese, the low level samples used in the previous collaborative study (see above) were considered satisfactory.

The new egg powder samples (here called II) were prepared as before by RIVM. But, poultry samples (II) were prepared by BioControl Systems, in the US, from naturally contaminated meat.

Table 5, page 7 of La Lettre de CECALAIT, gives the results obtained for both methods in both studies. They are expressed as the number of positive test results (taken from the paper to be issued in Journal of AOAC International, Feldsine et al., 2001).

Except for low levels in poultry I samples, both methods show the same performances.

➢SPECIFICITY STUDY

It was conducted by AFSSA Ploufragan, which applied the ISO 6579 draft method (without confirmation steps) to 100 to 200 *Salmonella* strains and to about thirty non *Salmonella*.

Usually specificity studies just deal with the detection or not of tested microorganisms. Nevertheless, the laboratory thought it would be more interesting to enumerate microorganisms after each step of the method *ie* pre-enrichment, enrichment using each of RVS and MKTTn broths and finally isolation using XLD and another agar.

Numerations were performed using Spiral plating onto plate count agar (PCA) after pre-enrichment and enrichment, then by ose plating onto 7 different isolation media, including mandatory XLD agar and the most used media during the first collaborative study.

134 *Salmonella* spp. strains were finally tested, representative of all *Salmonella* groups, with 46 different O serotypes and also some atypical strains. 8 *S* Typhi and 2 *S.* Paratyphi serovars were also included in the study.

After the pre-enrichment step, *Salmonella* spp., but also most of the other tested species grew well. Results obtained after the further steps are summarized up in table 6 in La Lettre de CECALAIT, page 8.

It shows, that despite the long and difficult procedure, this method allows a satisfactory detection of *Salmonella* spp. However confusion with other genera may occur, stressing the importance of confirmation steps and of skilled operators for this method.

However detection of *S.* Typhi and Paratyphi seemS even more difficult. The recovery of *S.* Typhi, affer enrichment in RVS broth is so low that detecting this species among other *Salmonella* is almost impossible. Enrichment in MKTTn broth seems better, but growth is still very low (in the former selenite broth, there was either low growth or survival). For *S.* Paratyphi, results are very different depending on the tested serovar : one is detected here, the other not.

6) CONCLUSION

At the end of these studies, horizontal reference method ISO 6579 (draft) has been found globally satisfactory for the detection of *Salmonella* spp., more difficult for the detection of *S.* Typhi and *S.* Paratyphi. Its precision performance appears equivalent to AOACI 995.20 & 2000.06 methods.So, it was recommended that AOAC International recognize this ISO method.

For ISO (and CEN), the following recommendations were drawn up and resolutions about them were taken at the meeting of the ISO standing Committe in charge of « Microbiology of food and agricultural products » in June 2001, in Bern :

- to include as an informative annex in the future standard, the precision data obtained in this project.
- to include, in the scope of the method, the following warning « The method may not recover all *S.* Typhi and *S.* Paratyphi ».
- to include the results of the comparison study with AOACI methods, to be issued in Feldsine et al., 2001, in the bibliographic references of the future standard.
- to go on with the revision procedure of the standard on the basis of this modified draft standard.

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