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French agency for food, environmental and occupational health safety

Maisons-Alfort laboratory for food safety

EU-RL CPS

European Union Reference Laboratory for Coagulase Positive Staphylococci

2010 Annual Report of the European Union Reference Laboratory for Coagulase Positive Staphylococci

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INTRODUCTION

In May 2006, the Maisons-Alfort laboratory for food safety of Anses (French agency for food, environmental and occupational health safety) –formerly AFSSA-LERQAP- has been nominated European Union Reference Laboratory (ex-CRL) for Coagulase Positive Staphylococci (EU-RL CPS, formerly CRL CPS), including *Staphylococcus aureus* and their toxins (see Regulation 776/2006).

The EU-RL CPS has undertaken the following actions in 2010, according to the actions planned at the 4th Workshop of the National Reference Laboratories (NRLs) (17&18 June 2010), as well as to the work programme defined in Annex I of the Framework Partnership Agreement between EC/DG SANCO and the EU-RL for the period 2006-2011.

Most of these activities aim at implementing, from an analytical point of view, the EC Regulation 2073/2005 on microbiological criteria for foodstuffs, modified by the Regulation 1441/2007, which includes in particular:

- 5 process hygiene criteria on CPS, defining a quantitative limit in:
  - cheeses made from raw milk or from heat-treated milk, ripened cheeses, and unripened soft cheeses,
  - milk/whey powder,
  - cooked crustaceans and molluscan shellfish.
- 1 food safety criterion on staphylococcal enterotoxins (SETs), requiring absence in 25 g in cheeses, milk/whey powder, to be tested when CPS enumeration is higher than 10^5 cfu/g when testing the above mentioned criteria on CPS.

NB: In brackets under each item, the scheduled duration of the action is indicated: either annual (limited to 2010), either multi-annual (on-going programme on several years).

1. DETECTION/ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI IN FOOD

Frame: The Standard methods EN ISO 6888-1 or 2 are cited as reference methods in the quantitative criteria of EC Regulation 2073/2005 for CPS.

1.1. STUDY OF SAMPLE TYPES USED FOR INTER-LABORATORY TRIALS

(multi-annual)

In the frame of the organization of future proficiency testing trial dedicated to the enumeration of coagulase positive staphylococci in dried milk powder, the EU-RL (Unit HMPA) has continued in 2010 the investigation study began in 2009 on the homogeneity and on the stability of the sample material for this type of matrix.

Different studies were conducted. Samples of milk powder were stored in different temperatures: 4°C, 8°C, 12°C and 20°C. Two levels of inoculation were tested. A satisfactory solution in terms of stability has not yet been found and the study needs to be further conducted in 2011.

1.2. PREPARATION OF TEST PORTIONS

(multi-annual)

In the Standard EN ISO 8261 *Milk and milk products – General guidance for the preparation of the test samples, initial suspensions and decimal dilutions for microbiological examination*, it is not specified how to sub-sample the test portion in the laboratory sample, depending on the different types of food matrices. This stage is however recognized as a major source of measurement uncertainty, in particular in solid matrices such as cheeses.

On 12 July 2010, the EU-RL (Unit HMPA) launched an enquiry to the NRLs on the sub-sampling of the test portions in cheese, in order to carry out an inventory of practices in the NRLs/Member States. 15 NRLs answered to the enquiry. The EU-RL is currently preparing the report of this enquiry, to be soon dispatched.

1.3. ENQUIRY ON THE SHELF-LIFE OF RPFA AGARS

(multi-annual)

On 5 May 2010, the EU-RL (Unit HMPA) launched an enquiry to the NRLs to carry out an inventory of the NRL practices regarding shelf-life of RPFA agars and to collect data on this aspect, as to be able to give guidance on the shelf-life of RPFA agars. 18 NRLs answered to the enquiry.

When discussing the outcome of the enquiry at the 2010 workshop, an ambiguity appeared on the purpose of the enquiry: the intended topic was the shelf-life of reconstituted RPFA, to be used as alternative to the confirmation step in EN ISO 6888-1.

Therefore, the enquiry was re-circulated to the NRLs in January 2011 in a corrected version with a clarified scope and available data were asked. 13 NRLs answered to the enquiry. The EU-RL is currently preparing the report to be soon dispatched.

1.4. METHODS OF ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI IN FOOD

(multi-annual)

In 2010, the EU-RL (Unit HMPA) launched a bibliographical study on alternative methods to enumerate CPS in food including molecular biology methods.

The EU-RL is currently preparing the report to be soon dispatched.

2. DETECTION OF STAPHYLOCOCCAL ENTEROTOXINS IN FOOD

Frame: The European screening method of the EU-RL CPS is cited as reference method in the criterion for staphylococcal enterotoxins (SEs) by the EC Regulation 2073/2005 modified.

2.1. INTER-LABORATORY PROFICIENCY TESTING TRIAL

(annual)

Since May 2008, repetitive problems were experienced with several batches of Transia Plate SE kits commercialised by BioControl Systems. Therefore the EU-RL (Team CAT-BAC) was obliged to postpone to 2011 the proficiency testing (PT) trial on the detection of staphylococcal enterotoxins scheduled in March 2010. This PT will be organised once the alternative methods to Transia Plate will be validated by an inter-laboratory validation trial prepared in 2010 and launched in January 2011 (see 2.9).

2.2. FEASIBILITY STUDY TO DEVELOP A PT MATERIAL OTHER THAN CHEESE

(annual)

A former study had been carried out to assess the feasibility of preparing materials made with food other than milk products for PT trials on staphylococcal enterotoxin (SE) detection. In that study, cooked ham purchased at a local store had been chosen as the matrix for PT material and the analyses had been performed with the Transia® Plate SE detection kit for SEA. The results were encouraging and have been sent to the NRLs for CPS by circular letter dated 30 March 2010. This preliminary study needed to be completed in order to cover a larger scale of SE types, SEA to SEE.

Because of the technical problems encountered with the Transia® Plate SE detection, other detection kits (Ridascreen® SET Total and Vidas® SET2) were tested in 2010 in an additional study. This additional study enabled us to assess the homogeneity and stability of the samples. A blank batch and five batches spiked respectively with SEA, SEB, SEC, SED and SEE were tested. The results of this study showed that the PT material made from cooked ham can be used for PT trials organized by the EU-RL for CPS to detect staphylococcal enterotoxins types SEA to SEE. The report of this additional study was dispatched by circular letter dated 12 October 2010.

2.3. EVALUATION OF COMMERCIALLY AVAILABLE ANTIBODIES AGAINST SE

(multi-annual)

The work begun in 2009 has been carried on in 2010 to evaluate other batches of commercially available antibodies and toxins. This study highlighted the fact that concentrations used for coating and/or revelation should be optimised from one batch to

another one. However, all the results obtained with the newly available batches were satisfactory, leading to the conclusion of the possibility to use commercially available antibodies to perform SEA to SED quantification in milk and milk based products.

Considering the development of an ELISA test against SEE, cross-reactions have been found between SEA and SEE showing a poor specificity of commercially available antibodies developed against SEE.

At last, the results obtained for the toxin type SEH were not satisfactory due to a lack of satisfactory antibodies.

2.4. USE OF MASS SPECTROMETRY FOR SE CHARACTERISATION AND QUANTIFICATION IN FOOD

(multi-annual)

This project is based on the development of an absolute quantification of SEs with a liquid chromatography/mass spectrometry (MS)-based methodology. In this approach, the sample is spiked with defined amounts of isotope-labelled protein(s) (PSAQ strategy). At first the PSAQ methodology has been developed in water samples and could be applicable for two types of staphylococcal enterotoxins: SEA and TSST-1.

In 2010, the aim of the work was to extend in water samples the use of the PSAQ method as a multiplex confirmatory method for six other SEs (SEB, SEC, SED, SEE and SEH). PSAQ standards and the quality control for the 5 above SEs have been produced by LEDYP (CEA, Grenoble, France) to develop a multiplex quantification of SEA, SEB, SEC, SED, SEE and SEH in water.

In 2011, LEDYP will produce and provide to the EU-RL (CAT-BAC Team) each of these standards, which will be used i) to implement the methodology in the CAT BAC Team and ii) to adapt and evaluate in the future the use of PSAQ on food matrices.

2.5. DEVELOPMENT OF CERTIFIED REFERENCE MATERIALS (CRMS)

(multi-annual)

For several years, the EU-RL and the associated NRLs identified a need for certified reference materials for staphylococcal enterotoxins in milk-based food.

In May 2010, DG SANCO has sent a letter to the EC Institute for reference materials and measurements (IRMM) in Geel (Belgium) for launching a possible collaboration with the EU-RL for CPS. In October 2010, Heinz Schimmel (IRMM) has contacted the EU-RL to exchange technical information in order to prepare a possible collaboration and to propose a trilateral meeting between DG SANCO, IRMM and the EU-RL.

2.6. DEVELOPMENT OF IMMUNO-QUANTITATIVE PCR FOR SE DETECTION IN FOOD

(multi-annual)

The EU-RL (Unit HMPA and Team CAT BAC) has continued to develop the immuno-quantitative PCR (iqPCR) method in 2010.

The sequential approach using a biotinylated anti-SE antibody linked to the biotinylated DNA with streptavidin component was tested with SEA. The assays concerning this approach were stopped because this approach was giving a poor sensitivity (0,1ng/ml for purified SEA toxin).

Then the method using the direct approach for the construction of the immuno complex (anti-SE-antibody/DNA conjugate) was also tested with SEA. The results showed that the method was not reproducible due to a degradation of the conjugate in the time.

The following strategy has been developed using an anti-sea biotinalyted antibody linked to an anti-biotin antibody – DNA conjugate in order to quantify SEA. The following steps were carried out in order to develop the Iq-PCR method with this approach:

- Optimization of the reagents (dilutants, washing buffers and concentrations of antibodies and conjugate).
- Determination of the sensitivity-standard curve for purified SEA toxin.
- Repeatability of the method on 10 assays.
- Specificity of the method against others major enterotoxins (SEB, SEC, SED, SEE).
- Limit of detection (LOD) and limit of quantification (LOQ) on purified SEA toxin.

The development on this method will be continued in 2011, and its limit of detection will be determined for culture supernatants as well as for food matrices.

2.7. NRL TRAINING

(multi-annual)

One training session dedicated to the SE detection: “detection of staphylococcal enterotoxins in milk and milk products and other matrices” was organised by the EU-RL, CAT BAC Team on 19-21 October 2010 with two participants: NRLs of Czech Republic (Olomouc) and Slovenia.

An overview on SE has been presented. Technical practice using the European screening method of the EU-RL for CPS, version 5, September 2010 has been provided. During the last day, the CEB Unit of EU-RL for CPS also presented an overview of the epidemiological monitoring and new molecular methods for characterisation and typing of CPS strains.

2.8. TECHNICAL AND SCIENTIFIC ASSISTANCE

(multi-annual)

In 2010, the EU-RL (Team CAT-BAC) developed a close collaboration with the Italian NRL for CPS. J.-A. HENNEKINNE and A. OSTYN (CAT BAC Team) participated to the Italian workshop for CPS on 8 April and to the food-borne disease congress on 9 April organised in Turin, Italy.

The CAT-BAC Team performed confirmatory tests further to positive results obtained by some NRLs with the screening method (ESM), under official controls performed according to the Regulation 2073/2005 modified.

Several NRLs sent contaminated samples to the EU-RL for CPS in order to know the type and the amount of toxin(s). 48 samples (food samples and/or concentrated extracts) have been received from the Italian NRL for confirmation and/or investigation of staphylococcal food poisoning outbreaks and SE quantification from cheese made for Italian PT trial.

Other samples have been received from the NRLs of Sweden (3), Cyprus (1), the Netherlands (2), Ireland (2) and Denmark (3).

2.9. IMPROVEMENT OF THE OFFICIAL METHOD

(multi-annual)

Frame: The European screening method of the CRL CPS is cited as the reference method in the criterion for SEs by the EC Regulation 1441/2007 which modified the EC Regulation 2073/2005.

Since May 2008, repetitive problems were experienced with several batches of Transia Plate SE kits commercialised by BioControl Systems, included as a detection kit in the European screening method of the CRL CPS (ESM). A temporary guidance on SE detection in food matrices and version 4 of the ESM (21 April 2010) have been sent to the NRLs for CPS and to DG SANCO (see circular letter dated 21/04/2010).

At the same time, the EU-RL for CPS investigated an alternative commercial kit to detect SEs in food. An intra-laboratory validation of the Ridascreen SET total kit (R-Biopharm) for detecting SEA to SEE in cheese has been performed. A scientific paper has been submitted in Letters of Applied Microbiology (LAM).

Moreover an intra-laboratory study has been conducted for detecting SEA to SED in other food matrices than milk products using the Ridascreen SET total kit and the Vidas SET 2 kit (bioMérieux).

Further to the satisfactory outcome of this intra-laboratory validation study, another updated version (version 5) of the European screening method of the EU-RL for CPS has been prepared and dispatched by circular letter dated 03/09/2010.

An interlaboratory trial for the validation of (i) the Ridascreen SET total kit (ii) the Vidas SET2 kit has been prepared in 2010 and was launched in January 2011 for detecting SEA to SEE respectively in food other than milk products. The outcome of this inter-laboratory study will enable to confirm the new version of the ESM.

3. CHARACTERIZATION AND TYPING OF STRAINS, EPIDEMIOSURVEILLANCE

Frame: In the DG SANCO support document to the call for the selection and designation of the new EU-RLs (SANCO/2214/2005), the Annex 1 describes the specific functions of the EU-RL CPS, which includes to keep abreast of developments in CPS epidemiology and to cooperate, as appropriate, with the Community structures involved into surveillance of CPS.

3.1. PROFICIENCY TRIAL ON SE GENES DETECTION BY MULTIPLEX PCR

(annual)

The EU-RL has organised during summer 2010 a PT trial dedicated to the *se* gene detection by multiplex PCR. 17 laboratories have participated to the trial. Preliminary results were dispatched to the participants on 3 March 2011.

3.2. DISPATCH OF STRAINS

(multi-annual)

Upon request of the Italian NRL and Slovakian NRL, the EU-RL (Unit CEB) has send *S. aureus* strains as positive controls for the PCR detection of staphylococcal enterotoxin (*se*) genes.

3.3. OPTIMISATION OF MOLECULAR TYPING BY PFGE

(multi-annual)

A new PFGE protocol was developed by the EU-RL, from different protocols already published. This protocol was successfully applied for the subtyping of 250 strains. The reference system *S.aureus* NCTC 8325 was used. A new BioNumerics database including the obtained PFGE profiles has been set up.

The optimized PFGE protocol was dispatched by circular letter dated 08/01/2010.

3.4. DEVELOPMENT OF SE GENES DETECTION BY MULTIPLEX PCR AND OTHER MOLECULAR TECHNIQUES

(multi-annual)

A. MULTIPLEX PCR

Further to the dispatch of the multiplex PCR protocol (by circular letter dated 23/12/2009), the EU-RL prepared and dispatched the validation report by circular letter dated 15/04/2010.

PCR detection of new gene *ses* and *set* was developed as an additional multiplex PCR, these additional PCR will be integrated in the *se* genes detection PCR protocol for dispatch in 2011.

A specific project was launched at the end of 2010 for standardization of PCR result interpretation through the BioNumerics software.

B. OTHER MOLECULAR TECHNIQUES

A new project was launched at the end of 2010 to conduct preliminary work on Real Time (RT) PCR detection of *se* genes. This project includes the implementation in the laboratory of RT PCR detection of all genes detected by the EU-RL multiplex PCR method. This RT PCR will be validated on a restricted panel of strains.

3.5. INVESTIGATION OF RECENT MOLECULAR SUB-TYPING TECHNIQUES

(multi-annual)

In 2010, the EU-RL has used *spa*-typing to characterize 180 *S. aureus* strains isolated from French staphylococcal food poisoning outbreaks. We also participated to the annual *spa* typing PT trial organized by the EU-RL Anti Microbial Resistance.

4. WORKSHOP OF THE NRLS

(annual)

The EU-RL organised the 4th workshop of the NRLs CPS on 17&18 June 2010, of general scope:

- to make a progress report on works undertaken by the EU-RL since the 2009 Workshop;
- to envisage the work programme for 2011 and further.

The report was dispatched by circular e-mail dated 20/08/2010.

5. VISIT TO NRLS

(annual)

In order to strengthen the link between the NRLs and the EU-RL, the EU-RL visits every year one or two NRL(s).

In 2010, Jacques-Antoine HENNEKINNE and Annick OSTYN visited the Italian NRL and met the network of the Italian reference laboratories (see 2.8).

6. TECHNICAL AND SCIENTIFIC ASSISTANCE TO THE EUROPEAN COMMISSION

6.1. DG SANCO ACTIVITIES

(multi-annual)

No specific request of DG SANCO in 2010.

6.2. PARTICIPATION TO CEN/ISO STANDARDIZATION ACTIVITIES

(multi-annual)

On behalf of the EU-RL and as EC representative, follow-up by the EU-RL coordinator (B. Lombard) of the activities of ISO/TC 34/SC 9[1] & CEN/TC 275/WG 6[2] for aspects related to the standardization of reference methods for CPS and SETs (1 jointed plenary meeting – budget EU-RL *L. monocytogenes*).

[1] Sub-Committee 9 « Microbiology » of Technical Committee 34 « Food products »

[2] Working Group 6 « Microbial Contaminants » of Technical Committee 275 « Food analysis – Horizontal methods »

In particular, participation to the works of one working group of ISO/TC 34/SC 9 of specific interest for the EU-RL activities and for DG SANCO: WG 3 “Method Validation”:

- mainly in charge of revising the Standard EN ISO 16140 on the validation of microbiological methods;
- 2 meetings: 11-12 January, Delft (NL), and 7-8 December, Brussels (BE).

7. PUBLICATIONS & REPORTS

7.1. REPORTS

- AFSSA/LERQAP/CAT BAC/2010/01. Interlaboratory comparative study on the performances of the Transia Plate SE batch number 09288-98 (March 2010).
- AFSSA/LERQAP/CAT BAC/2010/02. Feasibility study for the use of cooked ham as material for future interlaboratory trials on detection of staphylococcal enterotoxins (March 2010).
- AFSSA/LERQAP/CAT BAC/2010/03. Intralaboratory validation of the Ridascreen SET Total (R-Biopharm®) detection kit for staphylococcal enterotoxins SEA to SEE in dairy products (August 2010).
- Anses/Maisons-Alfort Laboratory for Food Safety/CAT BAC/2010/01. Second feasibility for the use of cooked ham as material for future interlaboratory trials on detection of staphylococcal enterotoxins
- AFSSA/LERQAP/HMPA/2010/01. 2009 inter-laboratory proficiency testing trial on the enumeration of coagulase-positive staphylococci.

7.2. METHODS

- European screening method for the detection of staphylococcal enterotoxins types SEA to SEE in milk & milk products and other food matrices, version 4 (21 April 2010). EU-RL for CPS (Unit CAT).

- European screening method for the detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices, version 5 (September 2010). EU-RL for CPS (Unit CAT).

7.3. ORAL COMMUNICATIONS AND SCIENTIFIC PUBLICATIONS

7.3.1. ORAL COMMUNICATIONS

- Ostyn, A. State of art on Coagulase Positive Staphylococci (CPS) and Staphylococcal Enterotoxins (SE). April, 9th, 2010 - Turin, Italy. Food borne disease congress.

- Hennekinne, J.A. Characterization of CPS strains and staphylococcal enterotoxins detection and quantification. April, 9th, 2010 - Turin, Italy. Food borne disease congress.

7.3.2. SCIENTIFIC PUBLICATIONS

- Ostyn, A., De Buyser, M.L., Guillier, F., Groult, J., Félix, B., Salah, S., Delmas, G., Hennekinne, J.A. (2010) First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. *Eurosurveillance*, 15, 13.

- Hennekinne, J.A., Ostyn, A., Guillier, F., Herbin, H., Prufer, A.L., Dragacci, S. (2010) How Should Staphylococcal Food Poisoning Outbreaks be Characterized? *Toxins*, 2, 2106-2116.

- Ostyn, A., Guillier, F., Prufer, A.L., Papinaud, I., Messio S., Krys, S., Lombard, B., Hennekinne, J.A. (2011). Intralaboratory validation according to the EN ISO 16140 Standard of the Ridascreen ®SET Total detection kit for a use in official controls of staphylococcal enterotoxins SEA to SEE in cheeses. *Lett. Appl.Microbiol*., accepted January 31, 2011.

- Hennekinne, J.A, De Buyser, M.L, Dragacci, S. Staphylococcus aureus and its food-poisoning toxins: characterization and outbreak investigation. *FEMS Microbiology reviews*. Submitted.

- Debuyser, M.L., Ostyn, A., Félix, B., Grout, J., Guillier, F., Hennekinne, J.A., Brisabois, A. (2010) Apport du génotypage de *Staphylococcus aureus* dans le diagnostic des toxi-infections alimentaires. *Bulletin épidémiologique de l'Anses*. Submitted.