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Editorial

Dear Readers and Authors,

After a two-year absence, we give you the latest issue of Euroreference. It is the 4th issue and will unfortunately be the last.

The new Euroreference format, launched in 2016 by ANSES with the support of 19 other institutions from 10 EU Member States, and dealing specifically with reference activities in the areas of animal & plant health, and food & drinking water safety, aimed to facilitate the sharing of experience and the development of scientific knowledge in the field of analytical reference work, both at the European and international levels.

After a healthy start and the active participation of scientific colleagues from several Member States, mobilisation has unfortunately dried up since 2018, despite the strong commitment of the directors of all the institutions involved. This new and last issue with only four articles (three of which are from ANSES) bears witness to this drop in contributions!

Driven entirely by a handful of ANSES staff with limited availability, and without any additional financial resources, the journal had set up a very simple editorial system to make it attractive to authors whose reference works were not eligible for inclusion in international scientific journals. Unfortunately, this model did not function, probably because many colleagues prefer to publish their research in journals that are more visible to the scientific community and therefore more rewarding for themselves and for their institutions.

We regret this, especially with regard to the reference activities in Europe, whose excellence is internationally recognised and which we remain convinced are essential to achieving a more robust and efficient system of health protection not only in Europe but also globally.

ANSES is therefore discontinuing this publication, at least temporarily. However, our conviction remains and we hope that new initiatives can be taken very soon so that a new network of institutions with reference mandates in the areas of animal & plant health and food & drinking water safety, can be set up and obtain sufficient resources to work and in doing so to stimulate European and international exchanges and collaboration in this field.

In the meantime, we sincerely thank all those who, at ANSES and at several other institutions, have strived since 2016 to make this experience work.

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Gilles SALVAT, Editor-in-Chief
ANSES Managing Director General in charge of
Research & Reference Division

Bruno GARIN-BASTUJI, EuroReference Executive Editor,
ANSES Strategy & Programmes Department

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Scientific and regulatory outcomes of the CEN Mandate in the microbiology of the food chain

Alexandre Leclercq[1], Bertrand Lombard*[2], Gwénola Hardouin[3]

Abstract

Reference methods in the field of microbiology of the food chain are standardised at the European level by the European Committee for Standardization (CEN) and at international level by the International Organization for Standardization (ISO). Historically, these methods were not fully validated. In 2010, the European Commission gave a mandate to CEN to fully validate a set of 15 methods covering the main bacteria, viruses, as well as bacterial toxins and metabolites, through inter-laboratory studies, and to standardise these methods or to revise the existing standards. The programme ended in July 2017 with the publication of the corresponding 15 CEN/ISO validated Standards. In addition, the *International Journal of Food Microbiology* recently published a special issue with the results of the validation studies that were conducted, and the European Regulation (EC) 2073/2005 on microbiological criteria was amended to include references to the new standards resulting from the CEN Mandate.

Keywords

✦ *Food Microbiology*

✦ Methods

✦ Validation studies

[1] Institut Pasteur, Biology of Infection Unit, National Reference Centre and WHO Collaborating Centre for *Listeria*, Paris, France

[2] ANSES, Laboratory for Food Safety, Maisons-Alfort, France

[3] AFNOR Standardisation, Saint-Denis, France

* Corresponding author : bertrand.lombard@anses.fr

Reliable microbiological methods in microbiology of the food chain represent an important tool for risk managers (public control authorities) and food business operators to ensure that safe food is provided to consumers. Reference methods applicable to the whole food chain, from primary production, food processing to the provision of food products to consumers, are standardised at the European level by CEN/TC 275/WG 6 and at the international level by ISO/TC 34/SC 9 . Both standardisation bodies work closely together as part of a CEN/ISO cooperation agreement to establish common CEN/ISO Standards in most cases. In the past, these standardised reference methods were based on experts' opinions and, sometimes, experimental data, but were not fully validated through inter-laboratory studies (ILS). A European project, SMT4-CT96-2098, conducted from 1997 to 2000 and financed by the European Commission (EC), made it possible to validate six standardised methods on the enumeration of *Bacillus cereus*, coagulase-positive staphylococci, *Clostridium perfringens*, *Listeria monocytogenes*, as well as on the detection of *L. monocytogenes* and *Salmonella* (Lahellec, 1998).

In 2010, EC gave a mandate to CEN, with € 3.1*m* of funding, (*i*) to validate a set of 15 reference methods in the field of food chain microbiology using ILS, and (*ii*) to standardise these methods, or to revise the existing standards, including the performance characteristics resulting from the validation studies. These methods were selected as reference methods in European Regulation (EC) 2073/2005 on microbiological criteria for food (Anonymous, 2005) for the microorganisms covered by existing criteria, or may be included at a later stage, because this regulation is regularly amended. This regulation is directly applicable to own-checks conducted by food business operators, but also indirectly to official controls defined in Regulation (EU) 2017/625 (Anonymous, 2017). These methods mostly deal with bacteria (*Campylobacter* spp., *Cronobacter* spp., *Enterobacteriaceae*, *Escherichia coli* O157, *L. monocytogenes*, *Salmonella* spp., *Vibrio cholerae-Vibrio parahaemolyticus*, *Vibrio vulnificus*, pathogenic *Yersinia enterocolitica*) but also viruses (norovirus, hepatitis A virus), bacterial toxins (*B. cereus* cereulide toxin, staphylococcal enterotoxins) and a bacterial metabolite (histamine). Each method has been validated and standardised in a CEN/TC 275/WG 6 task group led by a project leader, most of whom belonged to a EU Reference Laboratory for the microorganism in question, if relevant. The 15 validation trials were carried out by 150 laboratories in 35 countries, mainly in Europe but also in the USA. This CEN mandate ended in July 2017 with the publication of the 15 new or revised CEN/ISO Standards. In addition, this programme gave a sound basis for the development of the EN ISO 17468 Standard on the establishment or revision of a standardised reference method, with, in particular, the validation studies required (Anonymous, 2016).

This is the largest international programme to validate standardised methods in the microbiology of the food chain. Its scientific outcome led to the publication in January 2019 of a special issue of the *International Journal of Food Microbiology*, coordinated by Alexandre Leclercq. This issue compiles papers describing each validation study, written by their respective project leaders (*European and international validation of 15 main reference methods in the microbiology of the food chain*, 2019). The validation study on the detection of staphylococcal enterotoxins is expected to be published separately.

From a regulatory point of view, the significant outcome of this mandate was the recent publication of Regulation (EU) 2019/228 amending Regulation (EC) 2073/2005 (Anonymous, 2019). In particular, the reference to two new standards has been introduced: EN ISO 19020 for the detection of staphylococcal enterotoxins and EN ISO 19343 for the quantification of histamine. Other standards were already included in the regulation as undated references; their reference has been updated when necessary and their new version is automatically applicable. In addition, the expression of the qualitative limit in criteria has been modified from "absence in *x* g" to "not detected in *x* g", in accordance with the new versions of standardised qualitative methods.

Finally, the performance characteristics derived from the ILS conducted as part of this CEN Mandate provide essential information for (*i*) public authorities on the performance of the me-

thods used for official controls to check compliance with regulatory criteria; (*ii*) food business operators on the methods to use for their own checks; (*iii*) food microbiological laboratories on validated methods for their EN ISO 17025 accreditation (Anonymous, 2017) and (*iv*) validation/certification bodies to validate alternative commercial methods in comparison with fully characterised reference methods.

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The impact of disinfection stressors on *Listeria monocytogenes* in challenge testing of foods

Kristin Sæbø Pettersen *[1,2], Marina Elisabeth Aspholm[2], Yngvild Wasteson[2], Lena Haugland Moen[1], Taran Skjerdal[1]

[1] Norwegian Veterinary Institute, Department of Animal Health and Food Safety, Oslo, Norway

[2] Norwegian University of Life Sciences, Department of Food Safety and Infection Biology, Oslo, Norway

* Corresponding author : kristin.pettersen@vetinst.no

Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis (Schlech *et al.*, 1983; Cartwright *et al.*, 2013). The mortality rate can exceed 25% and listeriosis has the highest proportion of hospitalisation cases of all zoonotic diseases under EU surveillance (EFSA, 2017). *L. monocytogenes* infections are most commonly reported in people over 64 years old. The trend for an increase in foodborne listeriosis has been attributed to the rising proportion of older people, along with the higher consumption of ready-to-eat (RTE) food products (Cartwright *et al.*, 2013; EFSA, 2017; Rossi *et al.*, 2008).

According to the European Food Law (European Commission, 2005), the required sampling and analysis regimes are different for food products that support the growth of *L. monocytogenes* than for products that do not. Accordingly, European guidelines for conducting *L. monocytogenes* challenge tests were developed to determine whether a specific food product supports the growth of the bacterium (Beaufort *et al.*, 2014). Under these guidelines, *L. monocytogenes* should be acclimated to the temperature at which the experiment will be undertaken prior to inoculation of the food to shorten the lag phase during the challenge testing and to obtain maximum growth potential during the food's shelf life. These guidelines do not account for factors other than temperature influencing the physiological state of the inoculated strains.

L. monocytogenes contamination of RTE foods, heat-treated or not, usually occurs during food production, when the food is handled and/or is in contact with the production environment. Some *L. monocytogenes* strains can persist in food production facilities for years (Ferreira *et al.*, 2014) despite thorough cleaning and disinfection, conferring a continuous risk for contaminating production environments and food products. Persistent in-house strains are likely to be frequently exposed to washing agents and chemical disinfectants, particularly alkali treatments, which are frequently used to disinfect hard surfaces (Giotis *et al.*, 2010; Taormina and Beuchat, 2002b). The sub-lethal stress imposed by alkaline disinfectants may alter the resistance to subsequent stressors present in food products and in the human host (Giotis *et al.*, 2008; Giotis *et al.*, 2010; Segal *et al.*, 1981; Taormina and Beuchat, 2002a), resulting in altered growth potential.

Food production companies and researchers have used challenge testing to estimate the storage time up to a 100-fold increase in concentration of *L. monocytogenes*, and to assess the storage time before *L. monocytogenes* represents a food safety risk (De Cesare *et al.* 2018; Mejlholm *et al.* 2010; Pal *et al* 2008; Skjerdal *et al* 2010; Skjerdal *et al* 2014). According to the guidelines, the inoculum should be prepared to obtain immediate growth and maximum growth rate in the food products. However, an environmental contamination route, including stress imposed by a disinfectant, will alter the physiological state of the bacterial inoculum, compared with an inoculum grown under traditional laboratory conditions (Eom *et al.*, 2009). This may result in a prolonged lag phase for stressed bacteria compared with unstressed bacteria (Guillier *et al.*, 2005). If *L. monocytogenes* contamination arises from an environmental contamination route, the cells may require an extended time to recover, showing reduced growth potential in food products compared with an inoculum in good physiological condition, even if cold-adapted. The presence of environmental stressors may thus cause a discrepancy between naturally contaminated samples and challenge test data, consequently affecting food safety, food economy and food waste.

L. monocytogenes is characterised as a hardy agent, able to grow under anaerobic conditions, at high salt concentrations and under refrigeration temperatures (Chan and Wiedmann, 2009; Liu, 2008; Lorentzen *et al.*, 2010; Schirmer *et al.*, 2014). The bacterial growth rate in food can be decreased with stressors such as low pH, organic acids, nitrite, low water activity, background flora, modified atmospheres and cold temperatures (Mejlholm *et al.*, 2010; Mejlholm and Dalgaard, 2015b). These stress factors function as hurdles and are used separately

or in combination to reduce the growth rate of pathogens in food products.

Underestimations of pathogen growth in food products may lead to unacceptable high risks for consumers, but overestimation of growth may lead to unnecessary food waste. Considering the effect of relevant environmental stressors on the inoculum can lead to a more realistic *Listeria* shelf life without compromising food safety. In their review, Álvarez-Ordóñez *et al.* (2015) also pointed out that comparing the European guidelines with any alternative methodology is worthwhile.

The aim of this study was to evaluate how the inoculation procedure involving pre-exposure to a commercial chlorinated alkaline disinfectant influences the growth potential of *L. monocytogenes* in RTE food products. RTE chicken and RTE sliced deli meat were chosen as food model matrices because both products are widely used, have relatively long shelf lives and have been reported as food vehicles for *L. monocytogenes* transmission (Cartwright *et al.*, 2013).

Materials and Methods

■ *L. monocytogenes* strains and food products

Eight strains of *L. monocytogenes*, previously used in challenge tests to study growth in similar meat products, were used for inoculation of RTE food products (Skjerdal *et al.*, 2010). All strains are listed in Table 1. All these strains demonstrated rapid growth at 4°C in a previous study (Skjerdal *et al.*, 2010). Two laboratory strains, whereas Scott A was used as a reference strain, were included in the cocktail for both RTE chicken and RTE sliced deli meat. The respective cocktails further contained four isolates from similar food products and from food production facilities (Table 1). Because *L. monocytogenes* strains were selectively chosen in accordance with the specific product, statistical comparison of bacterial growth between these products was not performed.

TABLE 1 / Selected in-house *Listeria monocytogenes* strains for challenge testing.

ID number	Source	Used for inoculation of	Serotype*
VI 59788	Unknown product	RTE chicken	II c
VI 59789	RTE chicken	RTE chicken	II a
VI 59790	Sliced deli meat	RTE sliced deli meat	II a
VI 59791	Sliced deli meat	RTE sliced deli meat	II a
VI 59792	Meat balls	RTE chicken and sliced deli meat	II c
VI 59793	Wiener sausage	RTE chicken and sliced deli meat	II c
NVH-FMN	Laboratory	RTE chicken and sliced deli meat	IV b
Scott A	Laboratory	RTE chicken and sliced deli meat	IV b

*Serotype analysed by PCR (ANSES 2013, ANSES 2014)

RTE chicken and sliced deli meats were used as food matrices; they are heat-treated products with a commercial shelf life of 18 and 35 days, respectively. The package size was 200 g for the RTE chicken and 100 g for the RTE sliced deli meat. Food characteristics given on the product packages from the manufacturer are described in Table 2. In total, 369 packages of RTE chicken and 189 packages of RTE sliced deli meat from three different batches were included in the challenge test studies. All food packages were kindly provided by a private

food business company. Challenge tests with BHI broth (BactoTM brain heart infusion, Becton, Dickinson and Company, Sparks, MD, USA) were performed in parallel with the challenge tests using food matrices to confirm the ability of the *L. monocytogenes* strains to grow. A potential effect of stress was expected to be more readily detectable in RTE chicken than in the RTE sliced deli meat, because previous challenge tests of the latter product have documented only limited growth of *L. monocytogenes*.

TABLE 2 / Food characteristics provided by the manufacturer on the product package.

	RTE chicken	RTE sliced deli meat
Shelf life	18 days	35 days
Ingredients in 100 g	109 g chicken[a]	58% beef and pork
	Water	Water
	Salt (1.5%)	Salt
	Spices (including paprika)	Spices
	Glucose	Glucose
	Garlic	Onion
	Rapeseed and sunflower oil	Smoke flavouring
		Starch
Preservation	E262 (sodium acetate)	Anti-oxidant E315
	E325 (sodium lactate)	E325 (sodium lactate)
		E261 (potassium acetate)
		E326 (potassium lactate)
Nutritional content in 100 g	980 KJ	998 KJ
	25 g protein	12 g protein
	0 g carbohydrate	6.5 g total carbohydrate, including
	15 g fat	0.2 g sugars
		18.6 g fat, including 7.3 g unsaturated fat,
		8.5 g monounsaturated fat and
		2.4 g polyunsaturated fat
		1.7 g salt
Modified atmosphere	70% CO_2 and 30% N2	Yes, but not specified
Recommended storage temperature	0-4°C	0-4°C

[a] 109 g of raw chicken was needed for 100 g of the final chicken product

■ Preparation and characterisation of inoculum

The standard inoculation culture was prepared according to EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *L. monocytogenes* in RTE foods, Version 3 – 6 June 2014 (Beaufort *et al.*, 2014). In brief, strains were inoculated from frozen stocks into BHI broth and cultured separately at 37°C for 24 h. For pre-adaptation to cold, in accordance with the food storage temperature, 100 µl of each pre-culture was transferred into 9 ml of BHI broth and incubated separately for 7 days at 4°C. Following this adaptation, equal volumes of all *L. monocytogenes* cultures were pooled into a mixed culture and enumerated in accordance with part 2 of the horizontal method for the detection and enumeration of *L. monocytogenes* (Anonymous *et al.*, 2004). The mixed culture was immediately diluted in BHI and subsequently in physiological saline water (sodium chloride, Merck, Darmstadt, Germany) aiming for a *L. monocytogenes* concentration in the food of 100 colony forming units (CFU)/g when inoculating with 100 µl (Beaufort *et al.*, 2014). The dilutions in physiological saline water were applied to ensure equal and minimal carry-over concentrations of BHI between standard ino-

culum and the chlorinated alkaline-stressed inoculum.

For the challenge tests with disinfectant-stressed inoculum, standard cultivation methods were used, except that the bacterial suspension was not diluted because the subsequent exposure to stress was expected to reduce the level of viable bacteria. The chlorinated alkaline disinfectant Titan Hypo (Lilleborg AS, Oslo, Norway) was diluted in physiological saline water to a concentration of 0.5 or 1% after addition of 1 ml of the BHI containing bacteria. After 5 min exposure to disinfectant, 1 ml of the bacterial suspension was transferred to 9 ml of physiological saline water for dilution as suggested by Eom *et al.* (2009). Both the concentration and the exposure time were in accordance with the producers' recommendations for disinfection of environmental surfaces in food processing plants. A preliminary experiment revealed that physiological saline water and the solutions containing 0.5 and 1% of Titan Hypo had pH values (AOAC 981.12, 1982) of 6.9, 10.6 and 11.1 respectively. After adding 1 ml of BHI containing the inoculum, the pH changed to 7.2, 7.8 and 8.3, respectively. The final pH values in the inoculum samples were 7.2 for the control, 7.5 for the 0.5% solution Titan Hypo and 8.2 for the 1% solution of Titan Hypo.

■ Inoculation and storage of food samples

The inoculation was performed on the production date or the day after the production date, except for the last batch of RTE sliced deli meat, which due to logistical reasons were inoculated 5 days after the production date.

The product packages from three different batches were inoculated with 100 μl of the mixed cultures through a septum (ø 15 mm white, hard, PBI Dansensor A/S, Ringsted, Denmark) using a needle (0.6*25 mm) and syringe. The needle was used to spread the bacteria on the surface. The inoculated area of the food package was marked to facilitate later quantification of 25 g food matrix (De Cesare *et al.*, 2018). After inoculation, the inoculated food packages and control broth were constantly stored at 4°C until eight days after the expiry date.

The concentration of *L. monocytogenes* in the challenge test food packages and corresponding BHI tubes was assessed three times during the storage time, except for three biological replicates of RTE chicken and respective BHI tubes, which were sampled nine times during the storage period. A preliminary experiment revealed more rapid growth in RTE chicken than in RTE sliced deli meat, and the RTE chicken was therefore prioritised for more frequent sampling. Quantification of *L. monocytogenes* was performed according to a modified version of ISO 11290-2 using buffered peptone water (BPW, Oxoid, Hampshire, England) as diluent and agar *Listeria* according to Ottaviani Agosti (ALOA) and ALOA® supplement (Biomérieux, Marcy L'Etoile, France) as the agar medium (Anonymous *et al.*, 2004). BPW was used for optimal recovery of stressed cells. In each challenge test, the level of *L. monocytogenes* was determined from at least three biological and technical replicate samples. To ensure a minimal effect of the inoculation in modified atmosphere (MAP) conditions, the MAP was measured prior to inoculation and at three days post-inoculation (DanSensors MAP analyser, Ringsted, Denmark).

■ Characterisation of RTE chicken and sliced deli meat

Control samples from all test batches were initially analysed for natural contamination of *L. monocytogenes* using the ISO 11290-1 standard method for *L. monocytogenes* detection. The total aerobic count of the challenge test batches was analysed at the beginning and at the end of the storage period. Briefly, after blending, samples were diluted in unbuffered peptone water (UPW) (Becton, Dickinson and Company), plated on plate count agar (PCA) (Becton, Dickinson and Company) and the plates were incubated at 20°C for three days before counting colonies. The water activity (NMKL No. 168) and the pH (AOAC 981.12; 1982) were also analysed both at the start and at the end of the storage period. Additional single food packages from three different batches (n = 3) of RTE chicken and sliced deli meat were analysed for both extrinsic (MAP) and intrinsic (pH, water activity, dry matter, organic acids, NaCl and lactic acid bacteria) properties (Table 3). The analyses of pH, water activity, dry matter (NMKL No 23, 1991), organic acids (internal method of the commercial laboratory) and NaCl concen-

tration (internal method of the commercial laboratory using the chloride concentration and silver nitrate titration) were subcontracted to a commercial laboratory. The headspace MAP analysis in the food packages was performed using the PBI DanSensors CheckMate 9900 MAP analyser according to the manufacturer's instructions. The concentrations of organic acid in the water phase was calculated from the average dry matter weight and percentage of organic acid, using Food Spoilage and Safety Predictor (FSSP) software (Mejlholm, Gunvig *et al.* 2010, Mejlholm and Dalgaard 2013).

■ Statistical analysis

The food characteristic parameters were described with the mean and standard deviation (SD). Growth potentials were calculated from the log CFU/g difference of the median at the end and the median at the start (Beaufort *et al.*, 2014). The main aim of the study was to evaluate the effect of stress, not to categorise the food products in their respective growth category set by the regulations, and growth potentials below 0.5 log CFU/g were included in the regression analysis. The growth potentials were determined at the expiry dates and eight days after the expiry dates, because the sensory shelf life of the products were normally longer than the shelf life set by the food producer. The highest estimated growth potential among batches was used for final growth potential estimation of the specific product.

Statistical analyses were performed using Microsoft Excel 2010 and STATA, version 14. Multiple linear regression analysis was used to predict the growth potential, accounting for batch, level of stress exposure of the inoculum and inoculum concentration. The multiple regression model included predictors, which reached a p-value of < 0.2 in univariate regressions. The final model was determined by backward selection to obtain variables with p-values of < 0.05. Residuals were predicted and normality tested by using the Shapiro-Wilk test. Potential outliers were evaluated by their residual and leverage values.

Results

■ Growth potential of stressed and non-stressed *L. monocytogenes* in RTE chicken and sliced deli meat

L. monocytogenes strain mixtures were pre-exposed to either 0, 0.5 or 1% chlorinated alkaline disinfectant before inoculation of RTE chicken or sliced deli meat. The inoculum concentrations and the growth potentials of the *L. monocytogenes* strain mixtures in RTE chicken and sliced deli meat are given in Tables 4 and 5 and Figure 1. Growth of *L. monocytogenes* greater than 0.5 log CFU/g was observed for at least one of the challenge test repetitions within each category, except for the 0.5% stressed inoculum in RTE sliced deli meat.

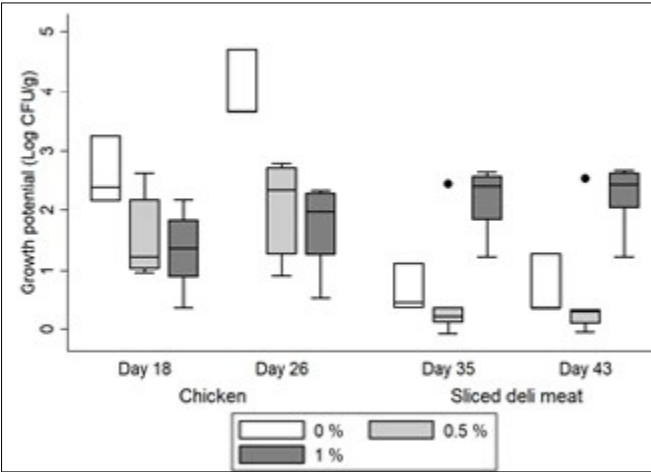### ■ Growth potential of *L. monocytogenes* in RTE chicken

Disinfectant exposure resulted in a concentration-dependent reduction in the growth potential of *L. monocytogenes* in chicken. A linear regression model, which included concentration of disinfectant and inoculum concentration as predictors for the growth potential estimations, revealed a significant effect of both inoculum concentration and the level of stress after 18 days of storage. The p-values were < 0.01 and 0.02, respectively, explaining 76% of the growth potentials (adj. R-squared). Potential batch variation had no significant effect on the growth potential for either of the products. A negligible batch effect is supported by the limited inter-batch variation observed for the food characteristics (Table 3). The inoculum concentration (p = 0.02) and the level of stress (p < 0.01) both had a significant effect on the final day of storage, explaining 70% of the growth potential estimates. There was an increased difference in growth potential in the RTE chicken between the stressed and non-stressed inoculum from the expiry date to eight days post-expiry.

TABLE 3 / Means and standard deviation (SD) of product characteristics for RTE chicken and RTE sliced deli meat, n=3.

Food characteristics	Batch	RTE chicken		RTE sliced deli meat	
		Mean	SD	Mean	SD
Total aerobic count, day 0 (Log CFU/g)	1, 2 and 3	1.75	0.23	1.91	1.08
pH, day 0		6.41	0.02	5.93	0.06
Water activity, day 0		0.98	0.00	0.97	0.01
Detection of *L. monocytogenes* in 25 g, day 0		ND[a]	ND	ND	ND
Total aerobic count, last day of storage (log CFU/g)	1, 2 and 3	6.86	0.37	7.07	0.66
pH, last day of storage		6.47	0.07	5.70	0.17
Water activity, last day of storage		0.98	0.00	0.97	0.00
Detection of *L. monocytogenes* in 25 g, last day of storage		ND	ND	ND	ND
Initial concentration of lactic acid bacteria (log CFU/g)	4, 5 and 6	1.70	0.19	1.90	0.44
NaCl (%)[b]		1.0	0.02	2.50	0.04
Dry matter (g/100 g)		34.9	0.67	37.2	0.59
pH		6.40	0.06	5.90	0.00
CO_2 % in headspace gas at equilibrium		55.0	0.91	2.30	0.26
N_2 % in headspace gas at equilibrium		45.0	0.92	97.7	0.28
O_2 % in headspace gas at equilibrium		0.1	0.01	0.1	0.01
Nitrite (ppm)		<0.16	-	4.50	1.85
Acetic acid (ppm)[b]		307	17	981	40
Citric acid (ppm)[b,c]		56	6	-	-
Lactic acid (ppm)[b]		12096	82	17224	159

a Not detected (ND).
b In the aqueous phase of the product
c Values below the level of quantification (LOQ = 20) increased due to matrix effects and were <59, <99 and <63 for the three tested samples of sliced deli meat

FIGURE 1 / Growth potentials of *L. monocytogenes* (log CFU/g) in RTE chicken and sliced deli meat at the expiry date (18 and 35 days, respectively) and eight days past the expiry date after exposure of the inoculum to 0, 0.5 and 1% chlorinated alkaline disinfection stress (n=3-5). Each box indicates the median (middle line in the box), the 25th (lower line of the box) and 75th (upper line of the box) percentiles, lower and upper adjacent value (single line) and outliers (dots)

Due to a potential compartment effect in solid foods, we compared the impact of disinfection on the growth parameters using BHI broth, representing more homogenous growth conditions than RTE chicken. The 1% stressed inoculum clearly demonstrated larger variance in *L. monocytogenes* concentration throughout the storage period, compared with the 0.5% stressed cells and the control (Figure 2).

■ Growth potentials of *L. monocytogenes* in RTE sliced deli meat

According to the linear regression model, disinfectant exposure resulted in a concentration-dependent increase in the growth potential in sliced deli meat (Table 4, 5 and Figure 1). In RTE sliced deli meat, stress and inoculum concentration had significant effects, with p-values of < 0.01 at both the expiry date and eight days past the expiry date and explained 89 and 86% of the observed growth potentials, respectively. One observation in the 0.5% stress exposed group for sliced deli meat (Figure 1) was defined as an outlier, because its residual value was 4.2 with a leverage of 0.8; it was therefore excluded from the regression model.

FIGURE 2 / Growth of a *L. monocytogenes* cocktail consisting of six strains (log CFU/g) in BHI broth and in RTE chicken from day 0 to day 26 (eight days post-expiry) after exposure of the inoculum to 0, 0.5 and 1% chlorinated alkaline disinfection stress (n=3-5). Each box indicates the median (middle line in the box) and the 25th (lower line of the box) and 75th (upper line of the box) percentiles. The high concentration of *L. monocytogenes* in BHI at day 26 may be due to sedimentation of cells.
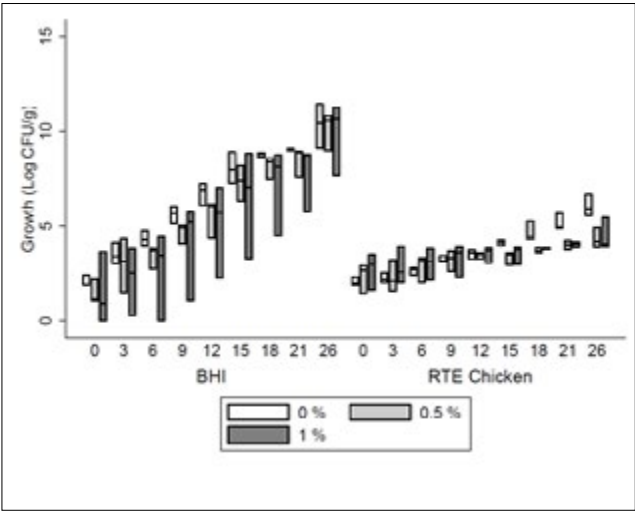
TABLE 4 / Challenge test data on RTE chicken.

Batch[a]	Desinf.[b]	Day 0[c]	Expiry date		Eight days past expiry date	
			Day 18[c]	δ[d]	Day 26[c]	δ[e]
1	0	2.3	4.5	2.2	6.0	3.7
2	0	1.9	4.3	2.4	5.6	3.7
3	0	2.0	5.3	3.3	6.7	4.7
1	0.5	1.5	3.7	2.2	4.2	2.7
2	0.5	2.7	3.7	1.0	3.9	1.3
3	0.5	2.9	3.9	1.0	4.9	2.0
3	0.5	2.8	3.9	1.1	5.4	2.6
3	0.5	1.0	3.6	2.6	4.6	0.9
3	0.5	3.3	4.7	1.3	6.1	2.8
1	1	1.7	3.8	2.2	4.0	2.3
2	1	3.5	3.9	0.4	4.1	0.5
3	1	3.0	3.9	0.9	5.4	1.3
3	1	2.0	3.8	1.8	4.2	2.2
3	1	2.0	3.8	1.7	4.3	2.3
3	1	3.6	4.5	1.0	5.4	1.8

[a] The original batch
[b] Level of chlorinated alkaline stress (%)
[c] Concentration on day 0, 18 and 26 (log CFU/g)
[d] Growth potential at expiry date (δ, log CFU/g)
[e] Growth potential eight days past expiry date (δ, log CFU/g)

TABLE 5 / Challenge test data on RTE sliced deli meat.

			Expiry date		Eight days past expiry date	
Batch[a]	Desinf.[b]	Day 0[c]	Day 35[c]	δ[d]	Day43[c]	δ[e]
1	0	2.2	2.5	0.4	2.5	0.4
2	0	2.4	3.5	1.1	3.7	1.3
3	0	1.9	2.4	0.4	2.3	0.4
1	0.5	3.3	3.6	0.4	3.6	0.3
2	0.5	3.8	4.1	0.2	4.2	0.3
2	0.5	4.0	4.1	0.1	4.1	0.1
2	0.5	1.5	3.9	2.5	4.0	2.6
3	0.5	3.8	3.7	-0.1	3.8	0.0
1	1	2.5	3.7	1.2	3.7	1.2
2	1	1.6	4.0	2.4	4.1	2.4
2	1	1.3	3.9	2.6	4.0	2.7
2	1	1.3	4.0	2.7	3.9	2.6
3	1	1.8	3.6	1.8	3.8	2.0

[a] The original batch
[b] Level of chlorinated alkaline stress (%)
[c] Concentration on day 0, 35 and 43 (log CFU/g)
[d] Growth potential at expiry date (δ, log CFU/g)
[e] Growth potential eight days past expiry date (δ, log CFU/g)

■ Characteristics of the food

Prior to inoculation, *L. monocytogenes* was not detected in any of the challenge test batches used. Regarding potential alteration of the MAP during inoculation, the inoculation procedure was evaluated as satisfactory, as shown in Table 6. The aerobic viable count increased in the challenge test batches of RTE chicken from an average ± standard error of the mean (SEM) of 1.8 ± 0.1 log CFU/g on the day of inoculation to 6.9 ± 0.2 log CFU/g at eight days after the expiry date (Table 3). In RTE sliced deli meat, the aerobic viable counts increased from 1.9 ± 0.3 log CFU/g on the day of inoculation to 7.1 ± 0.2 log CFU/g at eight days after the expiry date (Table 3). The water activity was consistent in both product types during storage and was considered to have a stable impact on *L. monocytogenes* growth during storage: 0.98 in the RTE chicken and 0.97 in the RTE sliced deli meat (Table 3). The pH was also stable in both products: 6.4 ± 0.0 and 5.9 ± 0.0 on the day of inoculation and 6.5 ± 0.0 and 5.7 ± 0 eight days after the expiry date in RTE chicken and sliced deli meat, respectively (Table 3). Additional characteristics of one sample, from three different batches of RTE sliced deli meat and RTE chicken, are also shown in Table 3. The level of dry matter was 34.9 ± 0.4% in the RTE chicken and 37.2 ± 0.3% (average ± SEM) in the RTE sliced deli meat (Table 3), and was used as input for the FSSP to calculate organic acid concentrations in the water phase of the products.

TABLE 6 / Control measurements of modified atmosphere packaging (MAP) 3 days after inoculation, means and standard deviation (SD), n =3, for CO_2, N_2 and O_2.

	CO_2 (%)		N_2 (%)		O_2 (%)	
	Mean	SD	Mean	SD	Mean	SD
RTE chicken, inoculated	56.1	0.5	43.9	0.5	0.0	0.0
RTE chicken, non-inoculated	53.8	0.6	46.2	0.6	0.1	0.1
RTE sliced deli meat, inoculated	2.6	0.4	97.3	0.4	0.1	0.0
RTE sliced deli meat, non-inoculated	2.4	0.1	97.5	0.1	0.1	0.0

Discussion

■ General considerations of the growth potential estimations

Maximising food safety and product shelf life and determining criteria for the withdrawal of foods require a correct estimation of the growth potential of *L. monocytogenes* in RTE foods at realistic production conditions. This study compared *L. monocytogenes* growth in RTE foods with a traditional inoculum preparation method and an alternative method including an environmental stressor, frequently used in food processing facilities. This new protocol for inoculum preparation was assumed to simulate a more realistic contamination route than the standard prepared inoculum for challenge testing. The "*Listeria* shelf life" of specific foods is often set as the time needed for a 2 log CFU/g increase in the *L. monocytogenes* level, based on the assumption that the concentration of *L. monocytogenes* is 1 CFU/g immediately after contamination. According to the guidelines for challenge tests (Beaufort *et al.*, 2014), the most important factors affecting *Listeria* shelf life and bacterial growth potential in food products are the properties of the inoculated strain(s), the inoculation level, the physiological state of the inoculated bacterial cell(s), the intrinsic properties of the food (*e.g.* pH, NaCl content, aw, associated microflora and antimicrobial constituents) and the extrinsic properties (*e.g.* time-temperature profile, gas atmosphere and moisture). The possible impact of these properties is assessed below.

All strains in the current study were either strains isolated from similar food products, or laboratory strains. Due to the food-matrix-specific composition of strains, to prepare a representative inoculum, only four of the strains were represented in both inoculums. Due to the different strains in the two products, the observed growth kinetic data for the two products cannot be compared and must be assessed separately.

As expected, the inoculation concentration significantly influenced the estimation of growth, and the inoculation concentration thereby had to be included in the linear regression analysis when analysing factors affecting growth potential. In contrast to naturally contaminated samples, where starting inoculum concentrations usually are below 1 log CFU/g (Beaufort *et al.*, 2007; Mejlholm *et al.*, 2015a; Pouillot *et al.*, 2007; Skjerdal *et al.*, 2014), the current study used starting concentrations between 1 and 4.0 log CFU/g. The European guidelines recommend a contamination level of 100 CFU/g in the food. However, one study claimed that the bacterial cell-to-cell variability has serious consequences for the challenge test design and that the inoculum concentration should be 3 log CFU/g to reach an acceptable level of variability and a consistent estimation of pathogen behaviour (Francois *et al.*, 2006). A low initial concentration of *L. monocytogenes* may also reduce growth potential due to lower ability to compete with the background flora and to the Jameson effect (Mellefont *et al.*, 2008). On the other hand, high inoculum concentrations may reduce the duration of the exponential growth phase, which may also lead to underestimation of growth potential (Francois *et al.*, 2006; Lardeux *et al.*, 2015). In the present study, the stressed inoculum concentration was challenging to standardise, despite preliminary studies and efforts to standardise the inoculum procedure. The protocol used in the current study may therefore introduce a bias to the growth potential estimates, as shown by the current results and by others (*e.g.* McManamon *et al.*, 2017). The stress exposure through the addition of a disinfectant agent in the current study likely introduced other caveats. However, removing any residual disinfectant agent by centrifugation prior to inoculation would not be representative of an environmental contamination route. Another alternative would be to use a reagent that chemically neutralises the stressor effect, but this is unrealistic for a natural contamination route and residuals from the neutralisers, which are not naturally present in the food industry plants, may potentially interact with the pathogen and the food matrix. Thus, it is difficult to make a stressed inoculum that contains a predictable concentration of bacteria and that is also representative of a realistic contamination route.

In addition to imposing increased stress on the inoculum, the disinfectants may have raised the pH of the food matrices, at least locally, which may have influenced the bacterial growth potential in the foods. If so, this pH effect can explain the contrasting effects of disinfectants in the chicken meat and in the sliced deli meat. Despite a 10-fold dilution of the disinfectant, disinfectant residues may interfere differently with the intrinsic qualities of the RTE chicken matrix compared with the sliced deli meat matrix. For example, the pH ranged by one unit between the stressed and non-stressed inoculum; thus the pH may change in the food matrix due to a carry-over effect of the alkaline disinfectant. The pH of chicken meat is closer to the optimum pH for growth of *L. monocytogenes* than the pH of sliced deli meat. A slightly increased pH due to the carry-over of disinfectants from the stressed inoculum could therefore lower the growth potential in chicken meat due to stress imposed on the bacteria, but give more favourable growth conditions in sliced deli meat due to a more favourable pH. However, higher pH in microenvironments in the inoculated samples due to carry-over of disinfectant was not possible to measure, because larger sample sizes are needed for pH measurements.

The decreasing growth potential in the RTE chicken may be due to a reduced physiological state of the bacterial cells after chlorinated alkaline stress (Eom *et al.*, 2009). Eom *et al.* (2009) reported that exposure to sodium hypochlorite affects both the lag-phase and the specific growth rate of *L. monocytogenes* strains. At 4°C, 75 ppm of sodium hypochlorite results in a higher specific growth rate than pre-exposure to 25 ppm, in broth and in a food matrix simulating crab meat (Eom *et al.*, 2009).

The food-specific intrinsic qualities also affect the behaviour of *L. monocytogenes* (Mejlholm *et al.*, 2015). An important difference between the RTE chicken and the sliced deli meat matrices is sodium nitrite, which is present in the sliced deli meat, but not in the chicken. Castellani and Niven (1955) showed that the bacteriostatic effect of nitrite was inversely proportional to pH. The chlorinated alkaline disinfectant may confer a local pH increase in the sliced deli meat during the inoculation procedure and thereby reduce the negative effect of nitrite and organic acids on the growth of *L. monocytogenes*.

The water activity and pH values were also consistent in both product types during storage. The variations in food characteristics in the current study are therefore not likely to provide any significant bias to the results.

■ Growth kinetics in RTE chicken versus BHI broth after stressing the inoculum

Due to the matrix effect in complex food products such as RTE chicken, it is also useful to analyse the effect of disinfection upon the growth in BHI broth, which is a more homogenous substrate. Therefore, RTE chicken and BHI broth were inoculated in parallel to compare the effect of disinfectant on growth of *L. monocytogenes*. The 1% stressed cells in broth clearly demonstrated larger variance in the concentration of *L. monocytogenes* than the 0.5% stressed cells and the controls throughout the storage period. The challenge of standardising the inoculum concentration when stressing the cells with disinfection was thereby further confirmed throughout the storage period (Figure 2).

■ Impact of sub-lethal stress on product categorisation according to EU microbiological criteria

According to the results from the current study, the level of stress imposed by the chlorinated alkaline disinfectant would not affect how the regulations categorise RTE chicken depending on growth potential. Except for one outlier in the 0.5% category of RTE sliced deli meat, all inoculated batches demonstrated *L. monocytogenes* growth levels of 0.5 log CFU/g or more, and are thereby categorised as "Ready to eat foods, able to support the growth of *L. monocytogenes* other than those intended for infants and special medical purposes" (Category 1.2) (European Commission, 2005). Nevertheless, the effect of disinfectant stress on the inoculum would affect how the food business operator would define a proper shelf life for this product.

The effect of chlorinated alkaline stress prior to contamination may have the potential to significantly alter the estimated concentration of *L. monocytogenes* in food products, thereby affecting food safety, food waste and the economy of the food industry. Further studies should be performed using single strains to determine the effect of stress imposed by disinfectants upon lag-phases and maximum growth rates of *L. monocytogenes* in food products.

Conclusion

The current study revealed significant changes in the growth potential of *L. monocytogenes* when the inoculum was pre-exposed to a commercial alkaline disinfection agent, consequently affecting the shelf life in terms of food safety. However, it is not clear whether the effect of the disinfectant was related to increased pH in the food matrix due to carry-over into the food matrix or to a change in the physiological condition of the inoculated bacteria. Furthermore, the significant impact of the inoculum concentration on growth potential and the challenge standardising the day 0 contamination levels indicate that the alternative method for inoculum preparation including stress is not recommended when performing traditional challenge tests with *L. monocytogenes* in RTE chicken and sliced deli meat.

Acknowledgements

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Comparison of the ISO method and three modifications of it for the enumeration of low concentrations of *Listeria monocytogenes* in naturally contaminated foods

Léna Barre*[1], Taran Skjerdal[2], Emilie Brasseur[1], Georgios T. Papageorgiou[3], Elena M. Odiatou[3], Hana Bulawova[4], Anais Auvolat[1], Sandra Favret[1], Bertrand Lombard[1] and Nathalie Gnanou Besse[1].

Abstract

Sensitive methods for enumeration of *Listeria monocytogenes* (*L. monocytogenes*) are needed to verify compliance with microbiological criteria in ready-to-eat foods. Here, we assessed the reference EN ISO 11290-2 method and three modifications of it with lower threshold levels for enumeration in terms of specificity, false results and practical limitations for use. Two of the methods, called the EURL and the Cyprus protocols, use membrane filtration to obtain a more concentrated test suspension, and the third, called the Norway protocol, uses less diluent. This study included 18 samples of foods naturally contaminated with *L. monocytogenes* at concentrations of 0.2-80 CFU/g. All four tested methods yielded valid results with good repeatability (Fisher's test, $p < 0.01$). The Norway protocol was the least laborious method and gave good results even for samples that could not be filtered.

Keywords

* Enumeration
* Filtration
* Food
* *Listeria monocytogenes*
* Microbiological methods

Acronyms and units used

* CFU/g: colony forming units per gram
* EURL: European Union reference laboratory
* ISO: reference method EN ISO 11290-2
* NRL: national reference laboratory
* RTE: ready-to-eat

[1] Université Paris Est, ANSES, Laboratory for food safety, EURL for *Listeria monocytogenes*, Maisons-Alfort, France

[2] Norwegian Veterinary Institute, Oslo, Norway

[3] State General Laboratory, Food Microbiology Laboratory, Nicosia, Cyprus

[4] State Veterinary Institute, Department of Food, Jihlava, Czech Republic

* Corresponding author : lena.barre@anses.fr

Introduction

Although its incidence is low, foodborne listeriosis causes serious symptoms and has a high fatality rate, up to 30% (Anonymous, 2000). Moreover, since 2000, the number of listeriosis cases has increased in several European countries (EFSA, 2007; EFSA 2012). For reasons not fully understood, this increase in infection appears to be linked to the increased use of ready-to-eat (RTE) products in which *L. monocytogenes* can grow during chilled storage. Several meat products and some seafood products belong to this category. Surveys of the incidence of *L. monocytogenes* in RTE foods at the retail level have been carried out during the past few decades. Across the EU, the estimated prevalence of foods with more than 100 CFU/g of *L. monocytogenes* at the end of shelf life is 1.7 and 0.43% for RTE seafood and meat products, respectively (EFSA BIOHAZ Panel, 2018). The majority of positive samples contain less than 1 000 CFU/g, but 2-4% contain 10 000 CFU/g or more at the end of shelf life (EFSA BIOHAZ Panel, 2018). Risk assessments from recent years have concluded that nearly all cases of listeriosis occur due to a very high dose of *L. monocytogenes* (1 000 to 1 000 000 CFU depending on the vulnerability of the consumer) after consumption of food that has been stored for a long time and/or at temperature abuse conditions (for review, see Buchanan *et al.*, 2016). Due to growth of the bacterium during storage, the concentration of *L. monocytogenes* in such products at the initial, processing stage may be below 10 CFU/g. Sensitive methods for the enumeration of *L. monocytogenes* in foods are therefore needed to demonstrate compliance with microbiological criteria and to limit the number of listeriosis cases.

The European and international standard method for enumeration of *L. monocytogenes* in food, EN ISO 11290-2 (ISO, 1998, 2004) is the reference method for *L. monocytogenes* in Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. The method is characterised by a theoretical limit of enumeration of 10-100 CFU/g in solid food. Its specificity has been improved with the introduction a more specific agar, Agar *Listeria* according to Ottaviani and Agosti (ALOA) (Gnanou Besse and Colin, 2004). However, this method still lacks sufficient sensitivity to determine bacterial counts in a precise manner at the limit of 100 CFU/g and to quantify the bacterium at even lower levels. Sensitivity of the ISO standard method can be improved by using more agar plates at the lowest dilution, but the feasibility of this approach is limited due to the costs of the selective medium and available space in incubators. Therefore, other alternative ways to obtain a more concentrated sample solution have been explored. (for review, see Gnanou Besse and Colin 2004, Välimaa *et al.* 2015; Jadhav *et al.* 2012). For routine analysis, however, it is important to ensure the same specificity and detection principle as the ISO standard method specified by the microbiological criteria. Here, we assessed three modifications of the EN ISO method that have been developed by reference laboratories, called the EURL, Cyprus and Norway protocols.

The EURL and the Cyprus protocols are based on membrane filtration followed by transfer of the filter to ALOA (AFNOR, 2009; Baudouin *et al.*, 2010; Barre *et al.*, 2015). The EURL protocol has been validated through an inter-laboratory study (Gnanou Besse *et al.*, 2008). With the analysis of a 5 g test portion of cold-smoked salmon, an enumeration limit of 0.2 CFU/g can be reached. The method has already been successfully used to monitor the growth and initial concentrations of *L. monocytogenes* in cold-smoked salmon (Gnanou Besse *et al.*, 2006). The method was recently evaluated for other food categories (meat, sausages, vegetables and seafood; Barre *et al.*, 2015), but this method proved non-applicable for some meat products. In light of this need for other protocols for meat products, the Cyprus protocol includes filtration, but the additives to the sample suspension are different to the EURL protocol, thereby representing an alternative for some matrices. The Norway protocol does not apply filtration to concentrate the sample suspension, but uses a smaller volume of diluent before homogenisation. Skjerdal *et al.*, (2014) used a 1:1 ratio for fresh salmon and diluent leading to a five-times more concentrated sample suspension than the ISO method and the-

reby a five-times lower detection level for enumeration. The protocol was used successfully for salmon, but not tested on other food matrices.

The objective of the present study was to assess the EURL, Cyprus and Norway protocols in terms of specificity, false-positive and -negative results and practical limitations for use compared with the reference method (EN ISO 11290-2) for the enumeration of *L. monocytogenes* in food, with focus on meat products, because the protocols have been tested on seafood products previously.

Materials and methods

■ Food samples and experimental design

The study was conducted in our capacities as the European Union and National Reference Laboratories for *Listeria monocytogenes*.

Samples naturally contaminated with *L. monocytogenes* (n=35) were kindly provided by the NRLs for *Lm* from various EU Member States and private laboratories. NRLs receive many samples, and the panel used in this study is therefore considered representative. Samples were received and stored frozen at -18°C. They were thawed the night before use at 3 ± 2°C.

The same suspension of a naturally contaminated sample was analysed with four methods, each repeated five times (see Figure 1) in parallel with the modified reference EN ISO 11290-2 standard method (current version at the time of the study), the EURL, Cyprus and Norway protocols (20 analyses in total per sample).

The reference method was modified by using a tryptone salt (TS) diluent and spreading 5 ml on 15 ALOA agar plates to increase its sensitivity. A maximum of 25 colonies per assay were confirmed.

### ■ Samples and sample preparation

The flow chart for sample preparation and all protocols tested are shown in Figure 1. Samples of 100 g were aliquoted into four 25 g portions to which tryptone salt (TS) diluent (25 ml) was added in a Stomacher bag fitted with a filter, then homogenised for 1 min using a blender (either a Smasher™, Biomérieux, France, or a Stomacher 400, Seward, West Sussex, UK) at normal speed. The homogenate (1:2 dilution) was transferred to flasks. Five ml from each flask was transferred to a new flask and used for the Norway protocol. The remaining homogenates (45 g per bag, sample and diluent included) were added another 180 ml of TS to obtain a 1:5 dilution, which together with the first dilution gives a 1:10 dilution. The contents in the flasks were combined and used as test suspension for the EURL protocol, the Cyprus protocol and the modified EN ISO 11290-2 protocol. Five sample preparations were carried out on each product.

■ EURL protocol for enumeration of *L. monocytogenes* using a membrane filtration method

The filtration method was carried out according to the protocol described by Gnanou Besse *et al.* (2008) with the same media and chemicals. Briefly, filtration was carried out using a standard commercial Pyrex apparatus, and a vacuum pump with a maximum vacuum power of 630-635 mm Hg (around 80-85 kPa) and an airflow rate of around 34 l/min. A 4.7 cm diameter and 0.45 µm pore-size membrane, composed of mixed cellulose esters and single-use filtration units with an effective 12.25 cm2 filtration area were used.

**FIGURE 1 /** Flow scheme of the protocols
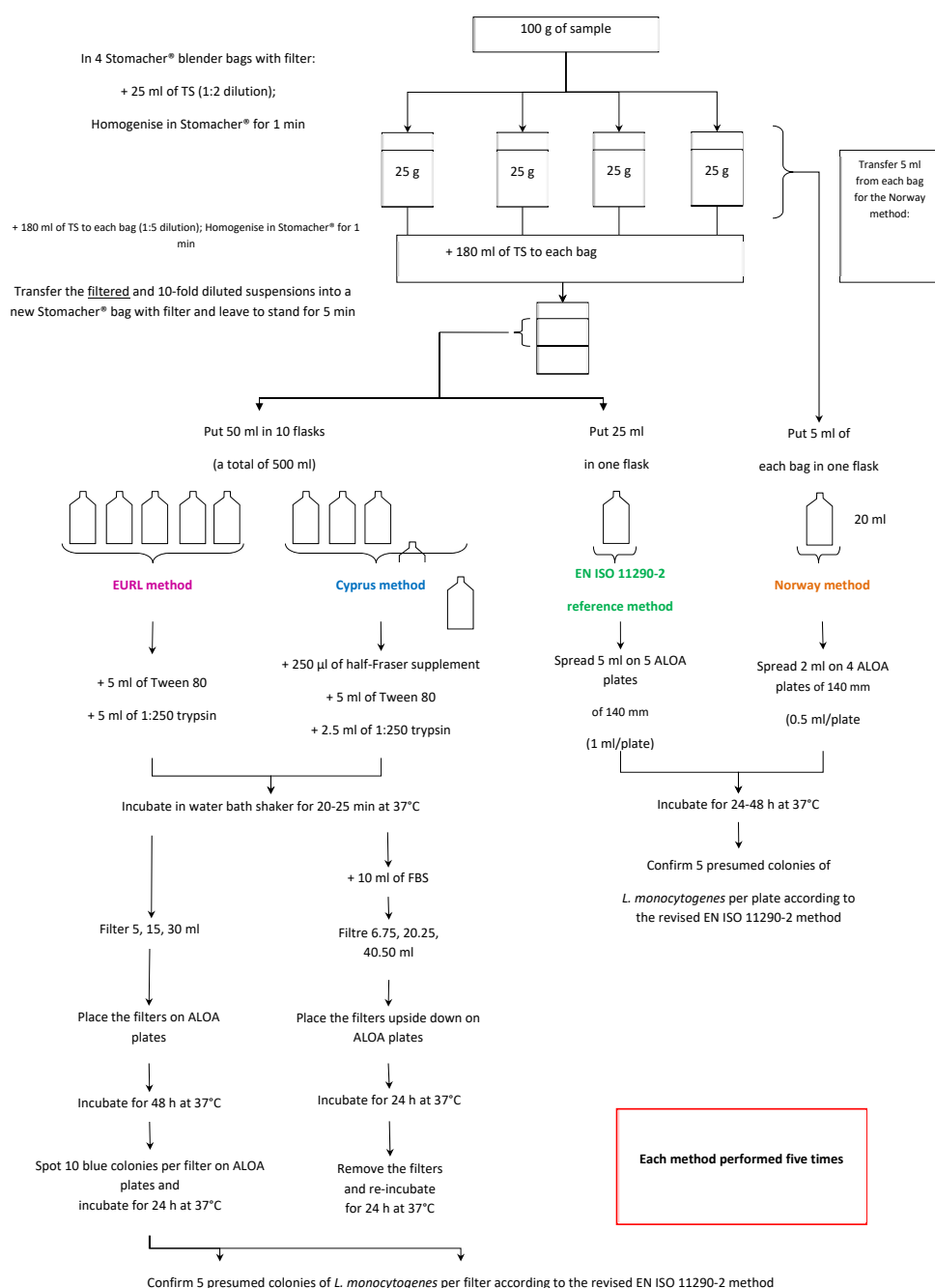
Three different volumes of the 1:10 diluted suspension (5, 15 and 30 ml) were immediately treated for a minimum of 20 and a maximum of 25 min at 37°C in a water bath shaker with 0.83% Tween 80 and 0.83% trypsin (addition of 1 ml of each reagent per 10 ml suspension to filter), and filtered. The procedure was repeated five times for each treatment. Every week, 10% trypsin from 1:250 stock solutions were prepared in phosphate buffer pH 7.5 containing 20 g dipotassium phosphate per litre of deionised water and stored at 4°C. The theoretical threshold of detection was 0.2 CFU/g.

Maximal filtration duration was set to 3-5 min. After this time, volumes of suspension which were not entirely filtered were considered as unfilterable. The filters were laid on ALOA plates. The plates were incubated upside down for 48 h at 37°C, and read after 24 and 48 h.

All *L. monocytogenes* colonies obtained on readable filters were counted. The volume analysed (corresponding to the selected filters) was recorded. *L. monocytogenes* colonies were blue without a halo, due to trypsin remaining on the filter. Consequently, in the present study, five typical blue colonies per filter were spot-inoculated on an ALOA plate, and incubated for approximately 6 to 18 h at 37°C to read the halo formation. Then, the typical *L. monocytogenes* colonies were confirmed according to the ALOA confirmation method.

FIGURE 2 / Comparison of enumeration results for *Listeria monocytogenes* obtained using the alternative methods (EURL, Cyprus (CY) and Norway (NO)) and the modified reference method (ISO) on naturally contaminated meat products (mean and standard deviation).
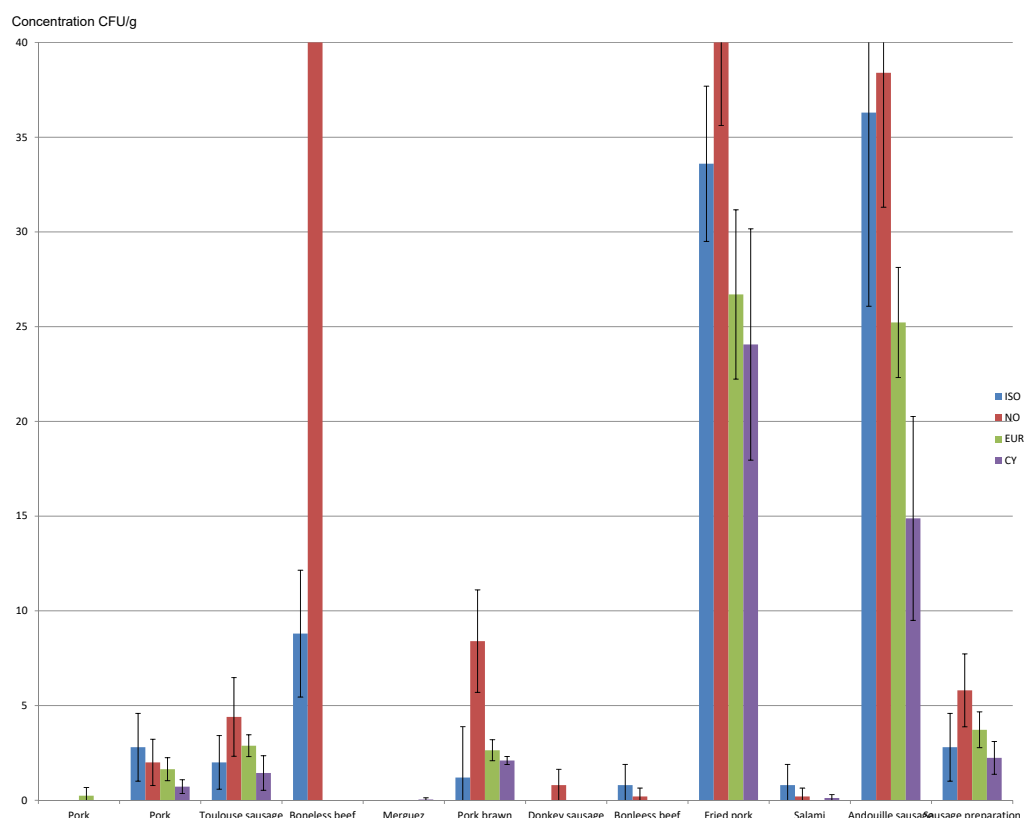
■ Cyprus protocol for enumeration of *L. monocytogenes*

The Cyprus protocol was similar to the EURL protocol with modifications intended to increase selectivity of the method. To reduce background microflora growth on the filter, half Fraser selective agents (Life Technologies, 10106169) were added to the suspension (0.5% final concentration). To favour better development of colonies and halo formation on ALOA agar without the need of subsequent inoculation, trypsin concentration was diluted with two parts of foetal bovine serum (FBS) that was added to neutralise trypsin activity before filtration (20% final concentration) (FBS, Oxoid, ref SR0166E) in order to obtain halo formation on ALOA agar. The filters were laid upside down on the selective agar and removed after 24h incubation at 37°C.

■ Norway protocol for enumeration of *L. monocytogenes*

The reference method was modified by using TS diluent and spreading 2 ml of a 1:2 food suspension on four ALOA plates of 140 mm (0.5 ml/plate) or 2 ml on six ALOA plates of 90 mm (0.3 ml, 0.3 ml, 0.4 ml/plate) to obtain a limit of enumeration at approximately 1 CFU/g. Confirmation of presumptive colonies were carried out according to EN ISO 11290.

■ Statistical analysis

Samples with either counts higher than 100 CFU/g or no colonies were excluded from the statistical analysis. The statistical analysis was performed according to the AFNOR NF V03-110 standard (AFNOR, 1998), which describes an intra-laboratory validation procedure for an alternative method compared with a reference method.

The Cochran test was used to check that the sample variances did not differ statistically and that the precision was stable over the scope of the method. The repeatability variances of the alternative and reference methods were compared using Fisher's test. The relative trueness of the alternative methods against the reference method was assessed by comparing the means with an error risk (α) of 1%.

FIGURE 3 / Comparison of enumeration results for *L. monocytogenes* obtained by the alternative methods and the reference method with other naturally contaminated products (mean and standard deviation)
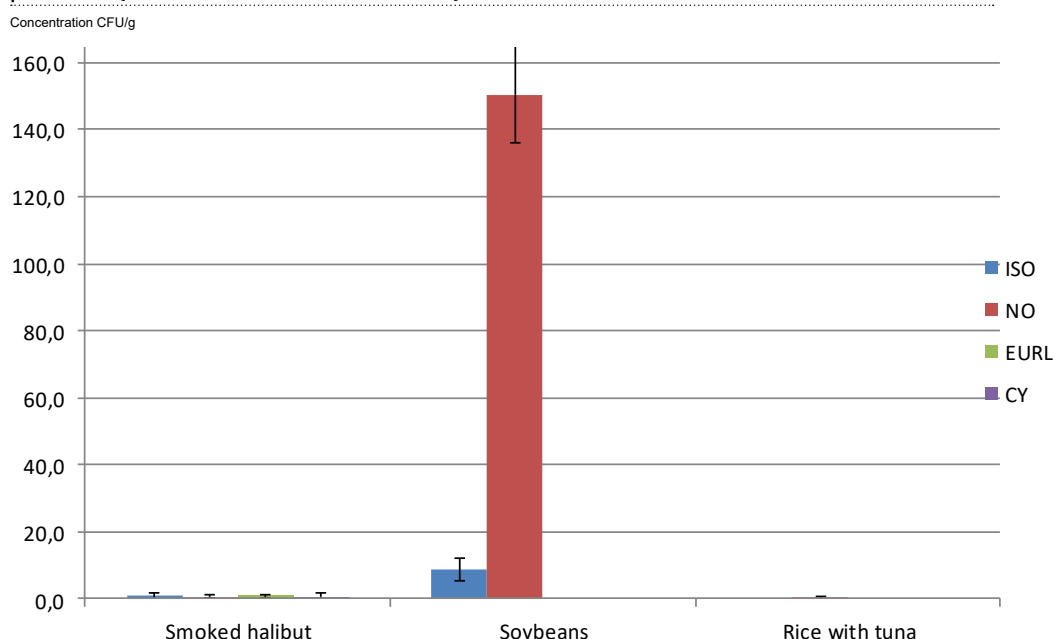
Results and Discussion

■ Applicability of the methods

Results are given in Table 1. Samples with *L. monocytogenes* concentrations greater than 1 CFU/g gave results in the same range for all methods. In most cases, the techniques made it possible to examine a larger quantity of food, thus greatly improving the sensitivity of the enumeration of *L. monocytogenes* in foods.

TABLE 1 / Result of the mean concentration (CFU/g) of *Listeria monocytogenes* obtained in each food sample, with the modified reference method analysing 5 mL and the alternative methods.

Samples	Method				
	ISO 11290-2, modified	EURL protocol	Cyprus protocol	Norway protocol	Filtration difficulties (filtration of 15 ml > 3 min)
	CFU/g (standard deviation)				
Pork	0	0.2 (0.4)	0	0	No
Pork	2.8 (1.8)	1.6 (0.6)	0.7 (0.4)	2.0 (1.2)	No
Toulouse sausage	2.0 (1.4)	2.9 (0.6)	1.4 (0.9)	4.4 (2.1)	No
Boneless beef	8.0 (3.3)	unreadable	unreadable	151.0 (14.7)	No
Boneless beef	0.8 (1.1)	unreadable	unreadable	0.2 (0.4)	No
Merguez	0	0	0.04 (0.1)	0	No
Pork brawn	1.2 (2.7)	2.6 (0.6)	2.1 (0.2)	8.4 (2.7)	No
Donkey sausage	0	unreadable	unreadable	0.8 (0.8)	Yes*
Fried pork	33.6 (4.1)	26.7 (4.5)	24 (6.1)	44.2 (8.6)	No
Smoked halibut	0.8 (1.1)	1 (0.5)	0.8 (0.9)	0.6 (0.5)	No
Salami	0.8 (1.1)	0	0.1 (0.2)	0.2 (0.4)	No
Soybeans	60.8 (12.0)	unreadable	unreadable	80.1 (18.9)	Yes*
Andouille (tripe) sausage	36.3 (10.2)	25.2 (2.9)	15 (5.4)	38.4 (7.1)	No
Sausage meat	2.8 (1.8)	3.7 (0.9)	2.2(0.9)	5.8 (1.9)	No
Rice with tuna	0	0	0	0.5 (0.4)	0.5 (0.4)
Sushi maki	0.1 (0.2)	0	Not tested	0	Yes*
Beef and mutton sausage	0.4 (0.4)	unreadable	Not tested	unreadable	No
Veal milanese	0.1 (0.2)	2.3 (3.2)	Not tested	1.8 (1.6)	Yes*

* Filtration difficulties indicate clogging of the filters in the EURL and/or the Cyprus protocols. Not relevant for the Norway and modified reference ISO protocol.

At *L. monocytogenes* concentrations below 1 CFU/g, the bacterium was detected with only some of the methods, and there was no clear pattern for any given method. The number of *L. monocytogenes* in the samples was likely too low to be homogenously present in samples for all protocols, and therefore it is not possible to determine which method was the most sensitive. In addition, four samples yielded unreadable results for both filter methods (EURL and Cyprus), but readable with the two others (reference and Norway). This discrepancy was due to either accumulation of the food matrix on the filters, or presence of bacteria that were able to grow on ALOA medium and cover the *L. monocytogenes* colonies (Baudouin *et al.*

2010). Given that the filter methods lead to a higher concentration of suspension than the other methods, the effect of background flora was more visible for these two methods. The samples with most overgrowth of background bacteria were generally products that included more ingredients. For the Norway protocol, in which the suspension was less concentrated than the filter methods, but more concentrated than in the reference method, only one sample gave unreadable results due to overgrowth (Table 1). The challenges observed with over-growth will be the same for any method based on identifying characteristic colonies on ALOA agar, e.g. the pour-plate method recently described by Hunt *et al.* (2017). Our observations also illustrate the importance of testing new methods on naturally contaminated samples with a realistic concentration of background flora.

Despite the caveats described above, the sensitive methods hold promise for the majority of the tested food samples. Practical limitations were mainly due to low filterability of the sample. Food matrices with high fat content or containing moisture-absorbing compounds such as flour, soybean and rice, were the ones with lowest filterability.

Seven of the samples used in this study were meat products typical of a specific region and/or prepared with several different ingredients, often cut in large pieces and pressed or fermented together. Such non-homogeneous matrices represent different niches, which in turn make it possible for more kinds of microbes to survive than in homogenous products. The likelihood of interfering background bacteria is therefore high. Analytical methods that are suitable for these kinds of product are likely to be suitable for more homogenous products as well, which indicates that the analytical methods studied here are suitable for more products than the ones tested.

■ Selection of data for statistical analysis

Thirty-five samples were analysed and we obtained 18 interpretable results (Table 1), 15 of which were analysed using all four methods. Data from three samples were analysed with three methods. These data were not included in the statistical analysis, but are given in Table 1 to illustrate the challenges and benefits of methods and food matrices studied. The non-in-terpretable results were either due to a concentration of *Listeria* too low to detect with any of the methods (64%) or overgrowth of background flora (36%).

Among the 18 results given in Table 1, 50% were interpretable for all the methods. About 40% of the samples were interpretable only with the plating methods (Norway and modified reference protocol) because (i) the concentration of background microbiota or of *Listeria* spp. was too high to enumerate characteristic colonies, particularly on the filter (see above), or (ii) the sample was not filterable. One sample was only interpretable for the modified reference protocol. About 10% of the samples were interpretable only using the filtration methods (EURL and Cyprus), because the concentration of *Listeria* was too low and no colonies were found with either direct plating method.

■ Comparison of the alternative methods with the reference method

For each interpretable sample, the mean of five *L. monocytogenes* counts is shown with standard deviations for all four protocols in Figures 2 and 3. The mean contamination levels of *L. monocytogenes* ranged from 0.04 to 150 CFU/g. According to the statistical analysis of data, both filtration methods and the Norway method yielded true results, according to the criteria set up in the statistical analyses, compared with the reference ISO standard method. The precision (repeatability) of all methods was as good as for the reference method (Fisher's test, $p<0.01$), and seemed to be better for the filtration methods: s_r^2 were respectively 3.7, 1.7 and 2.6 for the Norway, EURL and Cyprus protocols.

■ Assessment of each method

Both filtration methods showed good performance in terms of trueness and precision. The EURL method is sensitive, relatively rapid, easy to implement and cheap: to achieve the same sensitivity (e.g. the analysis of 50 ml of a 1:10 sample suspension) without filtration, up to 150 Petri dishes of selective agar would be necessary for each sample, spreading 1 ml on three 90-mm plates. Practicability is important to consider when choosing an analytical method. It includes ease of use, speed and cost. New methods including multiple steps, unusual materials and costly reagents may be difficult to implement in routine analysis. The filtration method is quite simple to use, but requires a specific apparatus and is more laborious than the reference method. However, the filtration part of the method is performed in a single step (no pre-filtration needed). Nonetheless, according to our experience in organising an inter-laboratory study, a very detailed protocol generally requires a training period before being able to use the method satisfactorily, particularly to overcome technical difficulties, such as filtering issues. The EURL method does not appear to be applicable to some food products, due to background microbiota that hinder colony reading on plates. Similarly, a previous study with naturally contaminated samples showed that the EURL method is not adapted for various meat products (Barre *et al.*, 2015).

The Cyprus protocol shares the same advantages/disadvantages as the EURL method, but was developed to obtain a clearer halo on ALOA agar. In the present study, no halo formation was observed below the filters with the EURL protocol, and re-plating to another ALOA plate was needed to obtain typical, visible blue colonies. Among the 12 samples that could be interpreted using the Cyprus protocol, halo formation occurred only in two samples, indicating that the protocol needs to be developed further. Addition of FBS in the ALOA medium and/or the washing of the membrane filter before plating with FBS are some options. In some cases, the overgrowth of the filters by background microflora was a disadvantage observed for both EURL and Cyprus methods, due to the large volume of the filtrate and the small diameter of the filters, making them inappropriate for some food matrices. The use of filters with a larger diameter or a lower volume of filtration, are possible alternatives to overcome this problem. Both filter protocols apply a 0.45 μm pore-size membrane, which allows some bacteria to pass. However, no sign of underestimation of *L. monocytogenes* was observed for these two filter protocols, and a smaller pore size would further reduce the filterability of the matrix.

The Norway protocol is rapid and easy; it requires fewer plates than the reference method to obtain the same limit of enumeration and it can be used for routine analysis regardless of the product. It also represents a good alternative to filtration protocols for some unfilterable food categories (Table 1). The enumeration level for the Norway protocol, 1-2 CFU/g depending on the number of plates used, is higher than for the filter protocols, but an improvement compared with the 10 CFU/g in the standard methods currently applied. A drawback of the method is its tendency for overestimation, even though the difference with other protocols was not significant in our study. Other studies in our laboratory (results not shown) with artificially contaminated salmon (N=20) and naturally contaminated heat-treated chicken meat (N=53) indicated a systematic overestimation of up to 50% with the 1:2 dilution compared with the 1:10 dilution. The difference is likely to be due to that the amount of dry, inert material in the suspension is five times higher in 1:2 dilutions and, as a result, the concentration of bacteria in the liquid fraction of the suspension higher. This bias can be corrected for by subtracting the dry weight of the sample in the calculations.

Conclusions

Despite limitations related to low filterability of samples containing fat or moisture absorbing compounds like rice and flour, the filter methods were better than the reference ISO standard method for the enumeration of low levels of *L. monocytogenes* in samples of other types of food products. Overgrowth of background bacteria was observed for all three alternative methods, but less frequently for the Norway protocol, which requires less diluent than the filter protocols. All methods showed satisfactory sensitivity, which is essential for implementation the European regulatory limit of 100 CFU/g as well as to conduct shelf-life studies and surveys for risk assessments at realistic conditions. The Norway protocol using less diluent to obtain a more concentrated sample was the least laborious one, and gave results as precise as the filter methods.

Acknowledgments

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Workshop on the risks associated with animal botulism and ANIBOTNET final meeting

Caroline Le Maréchal*[1], Fabrizio Anniballi[2], Luca Bano[3], Annica Tevell Åberg[4,5], Miia Lindström[6], Martin B. Dorner[7], Mikael Hedeland[4,5], Christian Seyboldt[8], Miriam Koene[9], Stefano Bilei[10], Yağmur Derman[6], Marianne Chemaly[1]

Abstract

A workshop on the risks associated with animal botulism was held in Maisons-Alfort, France in March 2019. The objectives were to debate the public and animal health risks related to botulism, to disseminate the results obtained from the ANIBOTNET project to other European research teams that were not involved in the project and to other stakeholders, and finally to strengthen and broaden the existing research network on this topic. The workshop was divided into 4 sessions: public and animal health risks associated with botulism, a specific focus on risks associated with agricultural biogas plants, dissemination of ANIBOTNET results, and network development and future perspectives. In all, 58 delegates from 13 countries attended the workshop. A summary of the main topics and discussions during the workshop is presented here.

Keywords

- Anaerobic digestion
- Animal health risks
- Biogas plants
- Botulism
- *Clostridium botulinum*
- Diagnosis
- ELISA

- Endopep-MS
- Epidemiology
- Foodborne disease
- Laboratory proficiency testing
- Next-generation sequencing
- One Health
- Public health risks

[1] ANSES, Ploufragan-Plouzané-Niort Laboratory, Hygiene and Quality of Poultry and Pig Products Unit, Ploufragan, France

[2] National Reference Centre for Botulism, Istituto Superiore di Sanità, Rome, Italy

[3] Istituto Zooprofilattico Sperimentale delle Venezie, Treviso, Italy

[4] National Veterinary Institute, Department of Chemistry, Environment, and Feed Hygiene, Uppsala, Sweden

[5] Uppsala University, Department of Medicinal Chemistry, Uppsala, Sweden.

[6] University of Helsinki, Faculty of Veterinary Medicine, Department of Food Hygiene and Environmental Health, Helsinki, Finlandy

[7] Robert Koch Institut, Berlin, Germany

[8] Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, Jena, Germany

[9] Wageningen Bioveterinary Research, Lelystad, the Netherlandsy

[10] Istituto Zooprofilattico Sperimentale delle Venezie, Treviso, Italy

* Corresponding author : caroline.lemarechal@anses.fr

Introduction

On March 28 and 29, 2019 a workshop on the risks associated with animal botulism was hosted by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) at its Headquarters in Maisons-Alfort, France. The workshop, funded by MedVetNet (2018_WS-1), included 8 presentations by invited speakers, 6 presentations by partners from the ANIBOTNET project, and 27 posters presented by delegates.

Background

Botulism is a neuroparalytic disease caused by botulinum neurotoxins (BoNTs), mainly produced by *Clostridium botulinum*. BoNTs affect both humans and animals worldwide. Botulism is included in list B of zoonoses and zoonotic agents in EU Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents (Authority *et al.*, 2018), i.e. the list of zoonoses and zoonotic agents to be monitored according to the epidemiological situation. Furthermore, BoNTs are included in category A of the US Centers for Disease Control and Prevention (CDC) list of potential biothreat agents (Centers for Disease Control and Prevention, 2012). The disease is rare but potential life-threatening in both humans and animals. Outbreaks in animals can be associated with high mortality and significant economic losses. On a worldwide basis, botulism is one of the most important diseases of migratory birds (Rocke, 2006). While surveillance data are available for humans, animal botulism is not listed among the 117 notifiable diseases monitored by the World Organization for Animal Health (OIE). In most European countries, animal botulism is not a notifiable disease, leading to a lack of animal botulism data regarding case numbers and outbreaks. However, a possible rise in the frequency of outbreaks was reported for France, Italy and some other European countries in the previous workshop focused on animal botulism that was held in Uppsala, Sweden in 2012 (Skarin *et al.*, 2013).

Objectives of the workshop

The purpose of this workshop was to discuss the public health risks associated with animal botulism. In addition, the workshop was used as an opportunity to share the results obtained from the ANIBOTNET project with other European research groups and stakeholders outside the project, and to strengthen and broaden the existing research network, fostering the One Health approach.

Animal botulism appears to be of increasing concern in some European countries (Skarin *et al.*, 2013), particularly in poultry flocks where it induces high mortality rates and economic losses, but also in other livestock productions including cattle, fur animals, and horses. Questions related to diagnosis, epidemiology, prevention, and control need to be explored to improve surveillance and management strategies. To address these questions, a 36-month (2016 – 2019) collaborative research project entitled ANIBOTNET was launched, with funding from the Animal Health and Welfare ERA-Net coordination actions.

The objectives of ANIBOTNET were to address knowledge gaps on animal botulism and to design strategies to improve surveillance, control, and prevention of the disease. The project aimed to improve an endopeptidase-mass spectrometry approach (Endopep-MS) to detect BoNTs in different types of matrices, standardize diagnostic tests, develop genotyping tools, develop knowledge about the epidemiology of the disease, and test vaccine and bio-control strategies.

The project involved 9 research groups from the EU (ANSES, IZSVe, IZSLT, ISS, FLI, RKI,

WUR, UH, and SVA) with complementary expertise in *C. botulinum*, BoNTs, mass spectrometry, veterinary diagnosis, genomic studies, epidemiology, and animal trials. It comprised five Work Packages (WPs). WP1 explored the stability, expression patterns, and regulation of BoNT type C, D, D/C and C/D genes in *C. botulinum* group III in vitro and in vivo. WP2 developed highly needed tools: Endopep-MS to detect BoNTs, Multiple Locus Variable Number of tandem repeats Analysis (MLVA) and MultiLocus Sequence Typing (MLST) for genotyping of *C. botulinum* isolates, and a workflow for mass spectrometric protein sequence analysis. WP3 aimed at better defining the epidemiological aspects of botulism. WP4 aimed at developing tools to consider strategies to prevent animal botulism. Finally, WP5 involved overall management.

The workshop delegates were risk management officers from local and national governments, veterinarians, scientists, laboratory technicians, PhD students, and company representatives from 13 countries: Belgium, Brazil, Denmark, Finland, France, Germany, Ireland, Italy, the Netherlands, Poland, Portugal, Sweden, , and the United Kingdom. An abstract book was drafted that included abstracts from the oral presentations as well as from posters that presented case reports or original research results related to botulism.

This report provides key information and summaries based on the presentations, discussions and abstracts provided in the abstract book.

Preamble of the workshop

ANSES Managing Director for Research and Reference Activities, Gilles Salvat, and Caroline Le Maréchal, Coordinator of the ANIBOTNET project and recipient of the MedVetNet workshop funding, opened the workshop. In addition to the presentation of the workshop programme, three recent outbreaks of botulism among wild birds in France in 2018 were presented and discussed. Importantly, they appear to have involved two different types of *C. botulinum* with type C/D and E neurotoxin genes detected by PCR. The detection of two different types of *C. botulinum* in three individual outbreaks among waterfowl raises questions for the first time about the emergence of additional risks to human health, as the identified type E is also pathogenic to humans.

Risks associated with botulism

The objective of this session was to discuss the human and animal health risks associated with botulism. This session started with a general overview of BoNTs and *C. botulinum*, covering recent original research related to mechanisms beyond BoNT production and *C. botulinum* sporulation, by Miia Lindström (University of Helsinki, Finland). To date, 7 BoNTs annotated as BoNT/A to G plus the recently described BoNT/X, and more than 40 subtypes have been described (Peck *et al.*, 2017). Six physiologically and genetically distinct clostridial species have been reported to be able to produce BoNTs: *C. botulinum* groups I, II, III and IV, *C. baratii* and *C. butyricum*. The botulinum neurotoxin is the most potent toxin known, with a human lethal dose potentially as low as 30–100 ng.

The session continued with a presentation by Cesare Montecucco (University of Padua, Italy) on the topic of non-clostridial species harboring the *bont* gene clusters. Some BoNT-like genes have been described in non-clostridial species such as *Weissella oryzae* (Mansfield *et al.*, 2015), *Enterococcus faecium* (Zhang *et al.*, 2018) or *Enterococcus* sp. (Brunt *et al.*, 2018), and *Chryseobacterium piperi* (Wentz *et al.*, 2017). The possible health risks related to these recently identified BoNT-like proteins were discussed during the presentation based on available knowledge: these toxins were identified with bioinformatic methods and were not associated with a disease or pathological status of any kind. It is not known whether these

genes are expressed or silent (Zhang *et al.*, 2017). Moreover, BoNT/En is not toxic in mice, suggesting mechanisms and targets different from those of the "classical" BoNTs.

The next presentation given by Mike Peck (QIB Extra Ltd, Norwich, UK) focused on human botulism, in particular foodborne botulism, which is the main form of the disease encountered in humans. Foodborne botulism is a severe intoxication caused by consumption of food containing BoNTs preformed by *C. botulinum*. It is a life-threatening disease with a mortality rate of 5–10 %, and has serious economic implications. Most foodborne botulism outbreaks are due to *C. botulinum* groups I and II producing BoNT types A, B, E and F. Group I and II strains are phenotypically very different, including growth requirements and heat resistance of spores, which suggests the need for informed risk assessment adapted to the characteristics of both groups, and the implementation of appropriate measures to prevent BoNT production by both groups. The application of safe production processes to prevent spore germination and further BoNT production is mandatory to ensure safe foods.

Luca Bano (Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Treviso, Italy) focused on animal botulism during his talk with a One Health perspective. Animal botulism is mostly due to BoNT types C, and D, and the mosaic forms C/D and D/C of *C. botulinum* group III. There are a few anecdotal reports on the involvement of these BoNTs in human botulism (Martrenchar *et al.*, 2019; Meyer *et al.*, 1953). Evaluation of the risk of contamination of farmed products during a botulism outbreak is a recurrent issue. Investigations conducted by Luca Bano on dairy farms during botulism outbreaks demonstrated that BoNT types C and D/C was not detected in tank or bovine udder milk. Botulism outbreaks are reported in wildlife, mostly wild birds and in livestock (poultry, cattle, minks, goats and sheep). In all, 40 outbreaks were confirmed by IZSVe between 2007 and 2018 in poultry, and 32 in cattle. A diagnostic strategy and epidemiological investigations were presented for both poultry and cattle production. Whole genome sequencing of strains involved in the outbreaks appears to be a reliable tool for epidemiological investigations.

The session ended with a presentation by Michel Popoff (Institut Pasteur, Paris, France) on the public health risks associated with botulism as a foodborne zoonosis. A review of available data on the possible transmission of botulism from animals to humans was presented, and the zoonotic potential of botulism, in particular during animal outbreaks with *C. botulinum* group III, was discussed. Only 11 cases of human botulism attributed to type C and one outbreak of botulism attributed to type D have been reported to date worldwide; however, unambiguous evidence is still lacking. BoNTs produced by *C. botulinum* group III have never been detected in human biological samples. Besides outbreaks, healthy carriage of *C. botulinum* by animals should also be taken into account. In most situations, human and animal botulism cases have distinct origins, and cross-transmission between animals and humans is a rare event. Considering the severity of this disease, human and animal botulism warrant careful surveillance. Effective identification and recording of animal outbreaks would help to better understand the epidemiology of botulism and putative links between animal and human cases. This is important when designing and targeting novel risk management measures[*].

Briefly, this session showed that botulism is a severe disease more commonly encountered in animals than in humans. In the majority of cases, the BoNT serotypes involved in human and animal outbreaks are different. Foodborne botulism due to improperly processed or stored food remains the main form of botulism encountered in humans in Europe, while the origins of contamination in animals are variable: contaminated feed, litter, wrapped bales, poultry manure, carcasses, etc. As healthy animals can be carriers of different types of *C. botulinum* (BoNTs A, B, E, and F), there is a clear zoonotic potential which resulted in including botulism as a zoonotic disease in the EU framework (Directive 2003/99/EC). However, the zoonotic potential of botulism in animals induced by *C. botulinum* group III (BoNT/C, D, C/D, and D/C) has been debated. Considering the available data, it can only be concluded that cross-contamination between animals and humans has never been demonstrated.

[*] Rasetti-Escargueil, C., Lemichez, E., Popoff, M.R., 2020. Public health risk with botulism foodborne zoonoses. *Toxins*,12, 17.

C. botulinum, botulism, and agricultural biogas plants

The second session of the workshop was dedicated to the specific topic of the fate of *C. botulinum* during anaerobic digestion in agricultural biogas plants. Anaerobic digestion has become increasingly important over the last few decades. This process was initially applied for the treatment of sewage, but more recently has been widely developed for the treatment of agricultural by-products in Europe. The fate of pathogenic bacteria, in particular *C. botulinum* through anaerobic digestion, has been poorly studied up to now, and public concerns regarding the outcome of anaerobic pathogens during this process and their potential development have been reported.

Ute Messelhäußer (Bavarian Health and Food Safety Authority (LGL), Oberschleißheim, Germany) opened the session by presenting the background and challenges related to anaerobic digestion and botulism. Using germ carrier experiments, Ute Messelhäußer's group showed that BoNT-producing clostridia cells were reduced with D-values between 1.0 ± 0.2 d at 55 °C and 34.6 ± 11.2 d at 38 °C during anaerobic digestion. The experiments also showed on the basis of a two-year survey of eight Bavarian agricultural biogas plants that no BoNT-producing clostridia could be detected among the 154 investigated samples (Froschle *et al.*, 2015b). Based on these results, it was concluded that the risk of encountering BoNT-producing clostridia in biogas digestates is very low if good agricultural practices are applied, and that the pathogen is even reduced in the biogas process (Froschle *et al.*, 2015a).

Lorine Derongs (IRSTEA, Rennes, France) and Caroline Le Maréchal (ANSES, Ploufragan, France) presented the results of a one-year monitoring programme of three French agricultural biogas plants for the detection and enumeration of *C. botulinum* using an optimized protocol. *C. botulinum* was detected in 33 % of manure and 79 % of digestate samples. *C. botulinum* type B was detected in all positive samples. Enumeration of *C. botulinum* in both matrices yielded very low concentrations (below 10 MPN/g) in all samples. This study suggested that *C. botulinum* may be present at very low levels in some manures or digestates, but is very unlikely to present a risk of growth during anaerobic digestion.

Axel Mauroy (Belgian Food Safety Agency, Belgium) concluded the session by presenting the Opinion 26-2017 of the Scientific Committee of the Belgian Federal Agency for the Security of the Food Chain. The objective was to evaluate the animal health risks associated with spreading of manure or digestates contaminated with *C. botulinum* type D (D/C) on farmed land. A qualitative risk assessment was carried out, according to the methodology recommended by ANSES. The risk for animal health when spreading contaminated manure or digestate was considered by the SciCom to be 'very low'.

It can be concluded from this session that anaerobic digestion does not induce the growth of *C. botulinum* during the process, and that the contamination risk associated with spreading digestate is similar to that with spreading manure.

Dissemination of the ANIBOTNET project results

After a brief introduction presenting the objectives and background of the ANIBOTNET project, Fabrizio Anniballi (Istituto Superiore di Sanità, Rome, Italy) started this session with an overview of animal botulism in Europe. Although the number of reported outbreaks has increased, the epidemiological situation concerning animal botulism in Europe remains unclear. A systematic review and meta-analysis was performed to improve knowledge on the current situation, and on the burden of animal botulism in Europe. However, the eligible articles and related data were limited to a few countries. To overcome the gap in epidemiological knowledge, a survey was launched by Fabrizio Anniballi's group to collect data across European countries, so as to obtain an overview of animal botulism in Europe.

Yağmur Derman (University of Helsinki, Finland) presented the results obtained in WP1. The data suggested that various primary sugar carbon sources differentially supported the growth, sporulation, and BoNT production of Group III *C. botulinum*. Experimental evolution studies showed that the probability of Group III *C. botulinum* losing its BoNT gene-carrying phage and thus becoming non-toxic varies significantly by host strain. Reinfection was not detected, suggesting that the laboratory conditions used support non-toxic states of these bacteria. WP1 also included the development of an experimental model for avian botulism. A poster presenting preliminary results from a non-toxic model was developed in WP1.

Annica Tevell Åberg (National Veterinary Institute, Uppsala, Sweden), Martin Dorner (Robert Koch Institute, Berlin, Germany) and Fabrizio Anniballi (Istituto Superiore di Sanità, Rome, Italy) then presented the outcomes of WP2 ("Development of tools intended for botulinum neurotoxin detection and genotyping"). This WP aimed at delivering validated diagnostic methods that can replace the traditionally used mouse bioassay for the detection of BoNTs. Endopep-MS is one of the most promising methods for BoNT detection available, and a strong candidate to replace the mouse bioassay. One objective of WP2 was to modify and validate Endopep-MS for matrices other than serum samples. A protocol for BoNT detection in cattle, horse, chicken, and turkey liver samples was developed and validated within the project. The new protocol was successfully used to confirm botulism in several botulism outbreaks. Another strategy tested within WP2 was ELISA, which is an easy-to-use method that does not require sophisticated equipment like Endopep-MS. Strategically, ELISA could be used as a screening method that can be easily applied by routine laboratories, while Endopep-MS could serve both as a screening and confirmation method performed in expert laboratories. Within WP2, ELISA tests for the detection of BoNT C, C/D, D, and D/C were developed and validated on veterinary samples previously analysed by PCR.

Protein sequencing of BoNT was performed by mass spectrometry within WP2, and PCR-based genotyping tools, i.e. MLVA and MLST were developed.

An interlaboratory proficiency test was also organised within WP2, in order to evaluate the different methods used for BoNT detection. The full outcomes of WP2 were presented in a poster.

Fabrizio Anniballi presented the epidemiological aspects of animal botulism (WP3). Strains collected during the surveillance activities conducted by the project partners were submitted to genetic comparison using the molecular tools developed in WP2 (MLVA and MLST), with the aim of evaluating their genomic variability. MLVA, MLST and whole-genome sequencing (WGS) were also used to identify the source of contamination in some outbreaks. With the aim of collecting epidemiological data on outbreaks occurring during the timeframe of the project, a specific database was built.

A talk presenting WP4 (Evaluation of measures to prevent and control animal botulism) was given by Luca Bano. Three different aspects were addressed in this WP: evaluation of the antibiotic resistance of strains, evaluation of a recombinant vaccine to elicit a protective response in different animal models, and evaluation of the potential of lactic acid bacteria as an antagonist of *C. botulinum* or BoNT production. The susceptibility of 71 *C. botulinum* group III field strains collected within five European countries to 13 antimicrobial drugs was tested during the project, with two main purposes: (i) first purpose of the test was prudent use of antimicrobials in poultry; and (ii) second purpose of the test was to explore the possibility of setting up a selective medium for the isolation of *C. botulinum* group III. The efficacy of a recombinant vaccine was tested in cattle, and the immune response was compared to the efficacy of a commercial toxoid-based vaccine. Finally, the efficacy of 40 lactic acid bacterial strains was tested against 37 *C. botulinum* strains. High sensitivity of *C. botulinum* to lactic acid bacteria was observed. All tested strategies showed promising results and opened up interesting possibilities for field applications so as to prevent and manage animal botulism outbreaks.

This session was closed by a discussion of WP5. This WP aimed at drafting guidelines intended for the management of animal botulism outbreaks: clinical suspicion, sample collection, laboratory confirmation, outbreak classification, data collection, therapeutic measures, and sanitation procedures. Each section was detailed during the presentation.

Future perspectives and networking

The workshop was closed by discussions about future projects, research topics, and networking. As illustrated by the proficiency test results from WP2, methods for BoNT detection, *C. botulinum* typing and sequencing, *C. botulinum* group III isolation, and botulism surveillance need to be better disseminated and adopted, and corresponding training provided.

This workshop was the second organised on the topic of animal botulism and allowed the number of research groups attending the workshop to be extended. Maintaining this network of laboratories in Europe or in other countries and including stakeholders appears to be very important to keep this research programme dynamic, and to make further progress on the management of animal botulism.

Overall, the workshop presentations and discussions gave in-depth insight into new available tools and progress on this topic. Replacement of the mouse bioassay, which is still the gold standard for BoNT detection, is a high priority. The ELISA and Endopep-MS methods were in-house validated for new sample materials, such as organs, during the ANIBOTNET project in the context of animal botulism outbreaks in real conditions. The proficiency test organised was a great opportunity to overcome the lack of quality control measures available in the field, and to test existing and novel diagnostic approaches on blinded samples. While many laboratories returned satisfactory results, the proficiency test also identified gaps and training needs that need to be addressed in the future. Important in vitro methods like Endopep-MS (Bjornstad *et al.*, 2014) or ELISA (Hansbauer *et al.*, 2016), if proper training is provided, can offer the same or better performance compared to the mouse bioassay.

The ANIBOTNET project and the workshop also highlighted particular topics considered to be priorities by the workshop delegates, and these should be addressed in the future:

- Continue research work initiated as part of the ANIBOTNET project, in particular validation of the alternative methods to the mouse bioassay in new matrices such as milk, and exploration of the mechanisms promoting group III *C. botulinum* growth and BoNT gene expression, with a view to better preventing outbreaks

- Share tools, diagnostic strategies, and guidelines for outbreak management, in particular with veterinarians

- Increase awareness on existing and emerging threats associated with animal botulism in order to obtain more reliable data on animal botulism

- Implement systematic characterization of *C. botulinum* isolates involved in animal botulism outbreaks, using WGS for example, to reinforce surveillance at the European level

- Collect data in order to identify and prevent risks to public health, the environment and the economy, for instance by collecting data regarding the contamination of food of animal origin produced during a botulism outbreak, such as milk, eggs, and meat, or by extending the studies on biogas plants to other European countries.

Future funding will help to maintain this unique network and to address the needs identified.

Acknowledgments

List of invited speakers

University of Helsinki	Miia Lindstrom
Quadram Institute	Mike Peck
Institut Pasteur of Paris	Michel Popoff
Istituto Zooprofilattico Sperimentale delle Venezie	Luca Bano
University of Padua	Cesare Montecucco
Bavarian Health and Food Safety Authority	Ute Messelhäußer
French National Research Institute of Science and Technology for Environment and Agriculture	Lorine Derongs
French Agency for Food, Environmental and Occupational Health & Safety	Caroline Le Maréchal
Belgian Food Safety Agency	Axel Mauroy

List of people involved in the ANIBOTNET project

ANSES: Caroline Le Maréchal, Marianne Chemaly, Rozenn Souillard, Sandra Rouxel, Typhaine Poezevara, Sabrina Macé, Laure Martin, Emmanuelle Houard, Patrick Fach, Cédric Woudstra

SVA: Kristian Björnstad, Mikael Hedeland, Ida Karlsson, Sofia Nyberg, Hanna Skarin, Annica Tevell Åberg

UH: Miia Lindström, Yagmur Derman, Cédric Woudstra

RKI: Martin B. Dorner, Martin Skiba, Ewa Schlereth, Heidrun Ranisch, Brigitte G. Dorner

WUR: Miriam Koene, Marc Engelsma, Yvonne Dijkstra

IZSVe: Ilenia Drigo, Tiziana Ferro, Angela Guolo, Elena Tonon, Luca Bano

FLI: Christian Seyboldt

IZSLT-ISS: Fabrizio Anniballi, Stefano Bilei, Concetta Scalfaro, Bruna Auricchio and Paola De Santis

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Contributing organisations

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail *French Agency for Food, Environmental and Occupational Health & Safety* www.anses.fr	Maisons-Alfort	France
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición / *Spanish Food Safety and Nutrition Agency* http://www.aecosan.msssi.gob.es	Madrid	Spain
AGES	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH/ *Austrian Agency for Health and Food Safety Ltd* www.ages.at	Vienna	Austria
APHA	Animal and Plant Health Agency www.gov.uk/government/organisations/animal-and-plant-health-agency	Addlestone / Weybridge	United Kingdom
BfR	Bundesinstitut für Risikobewertung *Federal Institute for Risk Assessment* www.bfr.bund.de	Berlin	Germany
EVIRA	Elintarviketurvallisuusvirasto *Finnish Food Safety Authority* www.evira.fi	Helsinki	Finland
FERA Science Ltd	www.fera.co.uk	York	United Kingdom
FLI	Friedrich Loeffler Institut *Federal Research Institute for Animal Health* www.fli.de	Riems	Germany
ISS	Istituto superiore di Sanità *Italian National Institute of Health* www.iss.it	Rome	Italy
IZSAM	Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise *Experimental Zooprophylactic Institute of Abruzzo and Molise* www.izs.it	Teramo	Italy
IZSLER	Istituto zooprofilattico della Lombardia e dell'Emilia Romagna *Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna* www.izsler.it	Brescia	Italy
LCV-SA	Laboratorio central de veterinaria - Laboratorio de sanidad animal / *Spanish central veterinary laboratory - animal health laboratory* www.mapa.gob.es/es/ganaderia/temas/laboratorios/lcv/	Algete / Madrid	Spain
NVWA	Nederlandse Voedsel en Warenautoriteit (Ministerie van Economische Zaken) / *Netherlands Food and Consumer Product Safety Authority (Ministry of Economic Affairs)* www.nvwa.nl	Wageningen / Utrecht	Netherlands
EPPO/OEPP	European and Mediterranean Plant Protection Organization Organisation européenne et méditerranéenne pour la protection des plantes www.eppo.int	Paris	France
PIWET (NVRI)	Państwowy Instytut Weterynaryjny. Państwowy Instytut Badawczy *National Veterinary Research Institute* www.piwet.pulawy.pl	Pulawy	Poland
SCIENSANO	Centrum voor Onderzoek in Diergeneeskunde en Agrochemie / Centre d'Etude et de Recherches Vétérinaires et Agrochimiques *Veterinary and Agrochemical Research Centre* www.coda-cerva.be	Brussels	Belgium
SCL	Service commun des laboratoires *French Ministries for Economy and Finances* www.douane.gouv.fr / www.economie.gouv.fr/dgccrf/Le-service-commun-des-laboratoires-DGDDI-et-DGCCRF	Paris	France
SVA	Statens Veterinärmedicinska Anstalt / *National Veterinary Institute* www.sva.se	Uppsala	Sweden
WBVR	Wageningen Bioveterinary Research http://www.wur.nl/en/Expertise-Services/Research-Institutes/Bioveterinary-Research.htm	Lelystad	Netherlands
WFSR	Wageningen Food Safety Research https://www.wur.nl/en/Research-Results/Research-Institutes/food-safety-research.htm	Wageningen	Netherlands

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