Food Anal. Methods (2016) 9:2979–2985
DOI 10.1007/s12161-016-0602-7

Validation of a Loop-Mediated Amplification/ISO 6579-Based Method for Analysing Soya Meal for the Presence of *Salmonella enterica*

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Received: 15 March 2016 / Accepted: 4 July 2016 / Published online: 19 July 2016

Abstract An alternative method for detection of *Salmonella* spp. in animal feed, based on the use of loop-mediated amplification (LAMP) in conjunction with a standard culturing procedure, was compared with the standard ISO 6579 as reference method, using soya meal as the test matrix. In the method comparison study, the sensitivities for both the alternative and reference methods were 100 %. The relative level of detection was 1.000. Tested against 100 *Salmonella* and 30 non-*Salmonella* strains, the LAMP-based method was 99 % inclusive and 100 % exclusive. The interlaboratory trial involved ten laboratories from eight European countries, testing eight samples at three contamination levels: 0 cfu/100 g, 1–5 cfu/100 g and 14–68 cfu/100 g. The trial specificity, or percentage correct identification of uncontaminated samples, was 96.3 % for both the reference methods and the LAMP/ISO 6579 alternative method, thus demonstrating its suitability for adoption as a procedure for rapid identification of *Salmonella* uncontaminated soya meal.

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Keywords *Salmonella* · Soya meal · Lamp · Validation · Alternative method

Introduction

Currently, analysis of animal feed for *Salmonella* is generally performed using the standard ISO 6579:2002 (Anonymous, 2002). ISO 6579 is based on bacterial culturing followed by biochemical tests. This enables the identification of uncontaminated samples in 3 days; any presumptive positive *Salmonella* isolated from a sample at that time are confirmed through further growth on selective media, and biochemical testing, in 7 days. To provide more rapid screening for *Salmonella*-free samples, a loop-mediated amplification (LAMP)-based method has been developed for the analysis of animal feed for the presence of *Salmonella* spp. (D'Agostino et al., 2015). LAMP is a nucleic acid amplification technique that operates isothermally without the need for complex thermocycling instrumentation (Notomi et al., 2000). The LAMP-based method is compatible with the standard

culture based method ISO 6579, as the first steps of both methods—pre-enrichment of the sample in buffered peptone water—are the same. This novel method allows identification of uncontaminated samples in 2 days, which would provide a significant time saving during routine use.

In the originating laboratory, the method of D'Agostino et al. (2015) worked consistently and efficiently. However, it is well known that the results of methods developed and published by one laboratory can sometimes be difficult to reproduce in other laboratories. The ability to reproduce results successfully in different laboratories is an absolute prerequisite for adoption of a detection method as a routine diagnostic tool (Rodriguez-Lazaro and Hernandez, 2016). Validation of any method should be necessary for its adoption as a standard (Jones and Marengo, 2016). The international standard procedure ISO 16140:2016 (Anonymous, 2016) can be used to demonstrate that the performance characteristics of a novel method are at least as good as those of a standard method, and thereby validate the newer method. Validation strictly according to ISO 16140:2016 is however very intensive and requires substantial resource. In this report, validation of the method of D'Agostino et al. (2015) against the method of Anonymous (2002) has been performed based partially on Anonymous (2016). This is the first reported validation of a LAMP-based method as an alternative to an existing standard method.

Materials and Methods

***Salmonella* Strains** Bespoke LENTICULE® discs were obtained from Public Health England (PHE; Colindale, London, UK), containing a low level of *Salmonella enterica* subsp. *Enterica* serovar Typhimurium (expected range of 1–5 colony-forming units [cfu] per disc). PHE also supplied a bespoke set of "blank" lenticules containing no microorganisms. Lenticules containing a medium level of *Salmonella enterica* subsp. *Enterica* serovar Typhimurium (expected range of 14–68 cfu per disc) were available as an "off-the-shelf" product from PHE. Other *Salmonella* serotypes were obtained from the Food and Environment Research Agency (FERA)'s in-house culture collection.

Inclusivity and Exclusivity Test Strains All non-*Salmonella* strains used in this study are listed in Table 3.

Animal Feed Soya meal was used as a representative animal feed. Soya meal was kindly supplied by John Thompson and Sons Ltd., Belfast, UK.

Detection of *Salmonella* Spp. in Animal Feed by ISO 6579 The reference method used was based on that described in "ISO 6579:2002 - Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp." with the following exceptions:

1. For primary enrichment of the sample, 100 g soya meal was added to 900 ml room temperature-buffered peptone water (BPW; Oxoid, Basingstoke, UK).
2. Secondary enrichment was performed using only the Rappaport-Vassiliadis medium with soya (RVS broth; Oxoid, Basingstoke, UK).
3. Plating out was performed using only xylose lysine desoxycholate agar (XLD; Oxoid, Basingstoke, UK).

Biochemical characterisation of presumptive positive colonies was performed using the API20e kit (bioMérieux, Basingstoke, UK).

Samples were considered as uncontaminated by *Salmonella* if, after examination of the selective agar plates, no typical or atypical colonies (as described in Section 9.4 of the standard) were present. Samples were considered as contaminated by *Salmonella* if any typical or atypical colonies which were present were confirmed as being *Salmonella* through biochemical testing as specified in the standard.

Loop-Mediated Amplification/ISO 6579-Based Method for Analysing Animal Feed for the Presence of *Salmonella* The alternative detection method was described in D'Agostino et al. (2015). Samples comprising 100 g feed were pre-enriched in 900 ml buffered peptone water, and then 2 × 100 μl aliquots were taken, one for secondary enrichment in a LAMP-friendly broth and the other for secondary enrichment according to ISO 6579:2002. A 3 μl aliquot of the LAMP-friendly culture was added directly into a LAMP reaction. The LAMP assay incorporated an internal amplification control (IAC); the target and IAC signals were distinguished by the difference in annealing temperatures of the amplicons. Samples were considered as uncontaminated by *Salmonella* if no target signal was obtained but a IAC signal was present indicating that no inhibition had occurred during the LAMP reaction. Samples were considered as presumptively contaminated by *Salmonella* if target signals were obtained; confirmation was performed by continuation of ISO 6579:2002, with biochemical testing (e.g. utilisation of citrate, fermentation of sucrose) performed using the API 20E kit.

Principle of the Validation

The principles of the validation were adapted from FDIS ISO 16140–2:2015. However, due to reasons of limited resource available within the study, adherence to the exact procedures was restricted. The study comprised two main parts: a method comparison study and an interlaboratory trial.

Method Comparison Study

1. A sensitivity study was performed to determine the ability of the LAMP/ISO 6579 method to detect the analyte. Anonymous (2016) recommends that naturally and artificially contaminated samples are used; however, in this study no naturally contaminated soya meal samples were available at the time, and therefore, artificially contaminated samples were used. Anonymous (2016) further specifies that at least three feed types within one category should be tested. However, only one type of soya meal was available for this study, and therefore, it was not possible to precisely follow the validation standard. To try to obtain fractional positive results as specified by Anonymous (2016), ten artificially contaminated samples at low level (1–5 cfu) and ten samples which had not been artificially contaminated ("Blank" samples) were tested. To artificially contaminate the samples, one low level lenticule was used per 100 g sample. The samples were analysed following the method as described in D'Agostino et al. (2015), which incorporates the reference method ISO 6579. Positive agreement (*PA*), negative agreement (*NA*), negative deviation (*ND*) and positive deviation (*PD*) values, the sensitivity of the alternative method (SE_{alt}), sensitivity of the reference method (SE_{ref}) and relative sensitivity (*AC*) were calculated as prescribed in Anonymous (2016). The false-positive ratio for the alternative method (*FP*) was not calculated as the method includes an integral confirmation step. The acceptability limit (AL) values were calculated using the formulae ($ND - PD$) and ($ND + PD$), as prescribed in FDIS ISO 16140–2: 2015.
2. A relative level of detection (RLOD) study was performed to evaluate the LOD of the alternative method against the reference method. Five blank soya meal samples, 20 low level contaminated samples (containing a mean of 4 cfu) and five medium level contaminated samples (containing a mean of 41 cfu) were analysed. To construct the low level contaminated samples, one low level lenticule was used per 100 g sample. To construct the medium level contaminated samples, one medium level lenticule was used per 100 g sample. The samples were analysed following the method as described in D'Agostino et al. (2015). The resulting data was analysed using the spreadsheet for calculating RLOD values available at http://standards.iso.org/iso/16140.
3. Inclusivity and exclusivity studies were performed to evaluate the ability of the alternative method to detect those strains (i.e. *Salmonella* strains) which would reasonably be expected to be detected by the reference method, and conversely not detect strains which are not expected to be detected by the reference method. One hundred pure cultures of different serotypes of *Salmonella* were tested and 30 pure cultures of non-*Salmonella* bacteria were tested. All strains were inoculated from stock cultures into 100 ml of appropriate liquid medium, and grown to an optical density of approx. 0.5 $O.D._{540}$. Serial dilutions were then prepared using phosphate-buffered saline, 1 ml of cell suspension containing approx. 10^2 cells was then inoculated into 900 ml buffered peptone water (no soya meal was added). Subsequently, the method of D'Agostino et al. (2015) was followed to completion.

Interlaboratory Study

This was performed following Section 5.2 of FDIS ISO 16140–2: 2015. Ten European laboratories—from Denmark, Greece (two laboratories), Ireland, Italy, Poland, Serbia, Spain and UK (two laboratories)—participated in the trials. The Food and Environment Research Agency (FERA) sent out ~3.0 kg soya meal and materials required for performing the method. Each participant also received eight blank lenticules, eight low level lenticules and eight medium level lenticules. Each lenticule was printed with a unique code, the identity of which was known by FERA but not revealed to the participants. Each participant was sent a detailed standard operating procedure (SOP) and a test report sheet on which to record the results and return to FERA for analysis. Each trial participant was required to prepare the media for the method. All ISO 6579:2002 media and reagents were purchased by the participating laboratory. The ingredients for the LAMP-friendly broth and the materials required for the LAMP assay were supplied by FERA. Eight 100-g samples of soya meal were spiked with one blank lenticule each, eight 100-g samples of soya meal were spiked with one low level lenticule each and eight 100-g samples of soya meal were spiked with one medium level lenticule each, and the samples were analysed following LAMP/ISO 6579-based method as described in D'Agostino et al. (2015). Four laboratories performed the LAMP assay using a GENIE II® (OptiGene Ltd., Horsham, UK) instrument, three of which were kindly supplied by OptiGene Ltd., and six laboratories performed the LAMP assay using a real-time PCR platform. The raw data were sent by

Table 1 Comparison of results of the paired sensitivity study of the reference method ISO 6579 for the detection of *Salmonella* in animal feed and the alternative method of D'Agostino et al. (2015)

Sample	Positive agreement (PA)	Negative deviation (ND)	Negative agreement (NA)	Positive deviation (PD)
Artificially contaminated	10	0	0	0
Blank	0	0	10	0

Table 2 Summary of the results for the various descriptors in the paired sensitivity study of the reference method ISO 6579 for the detection of *Salmonella* in animal feed and the alternative method of D'Agostino et al. (2015)

Descriptor	Formula	Value (%)
Sensitivity for the alternative method (SE_{alt})	((PA + PD)/(PA + ND + PD)) × 100	100
Sensitivity for the reference method (SE_{ref})	((PA + ND)/(PA + ND + PD)) × 100	100
Relative sensitivity (AC)	((PA + NA)/N[a]) × 100	100

[a] $N = 20$ (ten artificially contaminated + ten blank samples)

each laboratory to FERA. The data were statistically analysed according to Anonymous (2016). Parameters calculated were as follows: specificity for the reference method and the alternative method, and sensitivity for the reference method and the alternative method.

Results

Method Comparison Study

Sensitivity Study

All ten artificially contaminated samples, and all ten blank samples, were correctly identified by both the reference method and the alternative method. Table 1 shows the comparison of results.

Table 2 shows the summary of the results for the various descriptors in the study.

Table 3 Inclusivity and exclusivity of the LAMP/ISO 6579-based method for detection of *Salmonella enterica*

Inclusivity (target strains)	Test result
S. Aberdeen; *S.* Abony (2 strains*); *S.* Adelaide (2 strains*); *S.* Agbeni*; *S.* Agona ATCC 51957; *S.* Agona*; *S.* Ajiobo*; *S.* Alexanderplatz*; *S.* Amager*; *S.* Anatum*; *S.* Arecharaleta*; *S.* Bergen*; *S.* Binza (2 strains*); *S.* Blockley*; *S.* Bovis-morbificans*; *S.* Braenderup NCTC 5750; *S.* Braenderup*; *S.* Canstatt*; *S.* Caracas (3 strains*); *S.* Chailey*; *S.* Coeln*; *S.* Colarado*; *S.* Colindale*; *S.* Corvallis*; *S.* Cubana (2 strains*); *S.* Curacao*; *S.* Dresden*; *S.* Drypool*; *S.* Dublin (2 strains*); *S.* Duesseldorf*; *S.* Durban*; *S.* Eastbourne*; *S.* Ebrie*; *S.* enteritidis NCTC 4444; *S.* enteritidis NCTC 4777; *S.* enteritidis*; *S.* Florida*; *S.* Fresno*; *S.* Give (3 strains*); *S.* Goettingen*; *S.* Gold coast†; *S.* Grumpensis*; *S. Guinea*; *S.* Hadar*; *S.* Havana NCTC 6086 ; *S.* Houten*; *S.* Hvittingfoss*; *S. Indiana*; *S.* Isangi*; *S.* Istanbul*; *S.* Jukestown*; *S.* Kedougou*; *S.* Labadi*; *S.* Liverpool*; *S.* Livingstone NCTC 9125; *S.* London*; *S. Marina*; *S.* Matadi*; *S.* Mbandaka NCTC 7892; *S.* Mgulani*; *S.* Minnesota*; *S.* Negev*; *S.* Newington*; *S.* Mgozi*; *S.* Nairobi*; *S.* Oslo*; *S.* Ouakham*; *S.* Oxford*; *S.* Pakistan*; *S.* Pensacola*; *S.* Pomona*; *S.* Poona (2 strains*); *S.* Rissen*; *S.* Rubislaw*; *S.* Ruiru*; *S.* Saarbrueken*; *S.* Saint-paul*; *S.* Schwartzengrund*; *S.* Seattle*; *S.* Seftenberg NCTC 5788; *S.* Seftenberg NCTC 13385; *S.* Seminole*; *S.* Sorenga*; *S.* Stanley NCTC 0092; *S.* Stanleyville*; *S.* Stockholm NCTC 8488; *S.* Tees*; *S.* Tel-aviv*; *S.* Tel-el-kebir*; *S.* Tennessee*; *S.* Teshi*; *S.* Typhimurium NCTC 0074; *S.* Typhimurium NCTC 13348; *S.* Uganda*; *S.* Ugelli*; *S.* Vinohrady*; *S.* Virchow*; *S. Virginia*; *S.* Wandsworth*; *S.* Wangata*; *S.* Waycross*; *S.* Worthington*	Positive
S. enterica supsp. Arizonae NCTC 7301	Negative
Exclusivity (non- target strains)	Test result
*Acinetobacter baumannii**; *Actinomyces odontolyticus**; *Aeromonas hydrophila* NCTC 11195; *Bacillus cereus* NCDO 1771; *Brochothrix thermosphacta* NCDO 1676; *Carnobacterium pisciola* NCDO 2762; *Citrobacter freundii* NCDO 1516; *Clostridium perfringens* NCTC 10613; *Corynebacterium striatum**; *Cronobacter sakazakii* NCTC 11467; *Edwardsiella tarda* NCTC 10396; *Enterococcus avium* NCDO 2691; *Erwinia herbicola* NCTC 9381; *Escherichia coli* NCDO 1266; *Klebsiella aerogenes* NCTC 8172; *Kocuria rhizophila (*formerly *Micrococcus luteus)**; *Lactobacillus delbrueckii**; *Leuconostoc lactis* NCDO 533; *Listeria monocytogenes* NCTC 5214; *Pantoea agglomerans* NCDO 533; *Pediococcus pentasaceus* NCDO 990; *Peptostreptococcus indolicus* NCDO 2666; *Propionibacterium acnes**; *Proteus mirabilis* ATCC 35659; *Pseudomonas aeruginosa* NCDO 1525; *Saccharomyces cerevisiae* ATCC 24903; *Shigella sonnei* ATCC 25931; *Staphylococcus aureus* NCTC 8532; *Vibrio parahaemolyticus* NCTC 11344; *Yersinia enterolitica* NCTC 10460	Negative

*Field isolate

†Obtained from Public Health England

Table 4 Positive results obtained by the reference method (ISO 6579) in the interlaboratory study

Collaborators	Contamination level		
	L_0	L_1	L_2
Collaborator 1	0/8[a]	8/8	8/8
Collaborator 2	0/8	8/8	8/8
Collaborator 3	0/8	8/8	8/8
Collaborator 4	0/8	8/8	8/8
Collaborator 5	2/8	7/8	7/8
Collaborator 6	1/8	8/8	7/8
Collaborator 7	0/8	8/8	8/8
Collaborator 8	0/8	8/8	8/8
Collaborator 9	0/8	8/8	8/8
Collaborator 10	0/8	8/8	8/8
Total	3[b]	79[c]	78[d]

[a] Number of positive samples/number of total analysed samples

[b] Positive results at level 0 (P_0)

[c] Positive results at level 1 (P_1)

[d] Positive results at level 2 (P_2)

Relative Level of Detection Study

All five blank samples, 20 low level contaminated samples and five medium level contaminated samples were correctly identified by both D'Agostino et al. (2015) and ISO 6579. The RLOD was 1.000 with a lower 95 % CI of 0.300 and an upper 95 % CI of 3.337.

Inclusivity and Exclusivity Study

Table 3 shows the results obtained when the LAMP/ISO 6579-based method was tested on a range of *Salmonella* and non-*Salmonella* strains. The LAMP/ISO 6579 method was 99 % inclusive and 100 % exclusive.

Interlaboratory Study

All data from the participating laboratories were included as there was no instance where the results were obtained under inappropriate conditions, or the methods were not followed strictly. The results obtained by the individual laboratories are summarised in Tables 4 and 5.

The percentage specificity (SP) of the reference method and the alternative method (using the data after confirmation) were calculated, based on the results of level L_0 (Table 6):

The summarised results for all laboratories are given in Table 7.

Table 5 Positive results by the alternative method (LAMP/ISO 6579)

Collaborators	Contamination level					
	L_0		L_1		L_2	
	Presumptive positive	Confirmed positive	Presumptive positive	Confirmed positive	Presumptive positive	Confirmed positive
Collaborator 1	0/8[a]	0/8	8/8	8/8	8/8	8/8
Collaborator 2	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 3	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 4	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 5	2/8	2/8	7/8	7/8	7/8	7/8
Collaborator 6	1/8	1/8	8/8	8/8	7/8	7/8
Collaborator 7	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 8	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 9	0/8	0/8	8/8	8/8	8/8	8/8
Collaborator 10	0/8	0/8	8/8	8/8	8/8	8/8
Total	3[b]	3[c]	79[d]	79[e]	78[f]	78[g]

[a] Number of positive samples/number of total analysed samples

[b] Presumptive positive results at level 0 (P_0)

[c] Confirmed positive results at level 0 (CP_0)

[d] Presumptive positive results at level 1 (P_1)

[e] Confirmed positive results at level 1 (CP_1)

[f] Presumptive positive results at level 2 (P_2)

[g] Confirmed positive results at level 2 (CP_2)

Table 6 Percentage specificity of the reference method (ISO 6579) and the alternative method (LAMP/ISO 6579)

Descriptor	Formula	Value (%)
Specificity for the reference method (SP_{ref})	$(1 - ((C)P_0/N\_)) \times 100$	96.3
Specificity for the alternative method (SP_{alt})	$(1 - ((C)P_0/N\_)) \times 100$	96.3

$(C)P_0$ is the total number of false-positive results obtained with blank samples using the alternative method before and after additional confirmation. N_ is the number of all L_0 tests

Discussion

Alternative methods for *Salmonella* detection, based on the polymerase chain reaction (PCR), have been published (Delibato et al., 2014; Maurischat et al., 2014; McCabe et al., 2011), but this is the first report of a validation of a LAMP-based method for detection of *Salmonella*, as an alternative to an existing standard method. The study performed here is referred to in Anonymous (2016) as a "Paired" trial. This means that the 1° enrichment broth is the same for the reference method as it is for the alternative method. Due to resource limitations, it was only possible within this study to use one feed matrix, soya meal, for the validation; other molecular-based method validation studies using the approach of ISO 16140 have likewise used only one matrix (Delibato et al., 2014; Maurischat et al., 2014). In a previous study (D'Agostino et al., 2015), seven other animal feed types (corn gluten, distillers grains, rape meal, fish meal, microalgae biomass powder, organic full fat soya and whole rape) were successfully analysed by the LAMP-based method. The LAMP-based method validated on soya meal should be generically applicable to these other feed types.

In the sensitivity study performed during the method comparison study, the AL values of ND − PD and ND + PD were both zero, and thus below the parameters of 3 and 6 as prescribed in Anonymous (2016) for one category of feed type. The method of D'Agostino et al. (2015) is thus within the limits of acceptability for sensitivity as an alternative method to the use of ISO 6579 alone for detection of *Salmonella* in soya meal.

Ideally, to follow the method prescribed in Anonymous (2016) exactly, naturally contaminated samples would have been used, particularly for the sensitivity study. However, naturally *Salmonella*-contaminated soya meal was not available during this study, and therefore artificially contaminated samples were used instead (as has been done in other validation studies e.g. Delibato et al., 2014; Maurischat et al., 2014). Anonymous (2016) recommends (although does not mandate) that artificial contamination levels lower than 1 cfu are used, to achieve fractional recovery in the RLOD study. However, the lowest artificial contamination level in this study was 1 cfu (assuming that some of the low level lenticules contained this minimum quantity). Although therefore the validation standard was not adhered to precisely, it may still be concluded that using a very low artificial contamination level can reflect natural contamination situations. Therefore, with the RLOD in this study being 1.000, the method of D'Agostino et al. (2015) can reasonably be considered to be equivalent to the standard method with regard to their respective levels of detection of *Salmonella*.

One *Salmonella* strain (*S. enterica* subsp. *arizonae*) failed to amplify during the inclusivity testing, thus affecting the specificity value. The reason for this failure is unknown. The LAMP assay used the primers designed by Hara-Kudo et al. (2005); these authors found that they were able to mediate the detection of all seven strains of *S. enterica* subsp. *arizonae* which they tested. Although it has been occasionally isolated from food (Hall and Rowe, 1992), *S. enterica* subsp. *arizonae* is not a common foodborne *Salmonella* and is usually associated with those who handle reptiles or travel abroad (Di Bella et al., 2011). There are no reports of this bacterium being isolated from animal feed.

In the interlaboratory study, it was decided for reasons of cost and logistics not to send out *Salmonella*-contaminated soya meal samples which were prepared in the originating laboratory. The use of commercially available certified *Salmonella* reference materials was considered advantageous, as they were supplied directly to the participants by the manufacturer, and as they were stabilised materials it reduced the possibility of fluctuating temperatures during transit affecting the viability thus introducing unacceptable variation into the trial. On the other hand, the natural variation created by the participants' preparation of the growth medium is an advantage, as the validation protocol (ISO 16140) recommends natural variability during the trial in order to provide more robustness to the data.

During the interlaboratory trial, one laboratory reported two false-positive results and another laboratory reported one false-positive result. However, there was no evidence of deviation

Table 7 Summarised results for all laboratories for the paired study validation of the LAMP/ISO 6579-based method for detection of *Salmonella* in animal feed

Level	Number of samples tested (N)	Positive agreement (*PA*)	Negative agreement (*NA*)	False negative (*FN*)	False positive (*FP*)	Positive deviation (*PD*)
L0	80	3	77	0	0	0
L1	80	79	1	0	0	0
L2	80	78	2	0	0	0

from the protocol or inappropriate performance of the method, and since each of these laboratories reported an identical number of false-negative results it was considered likely that errors were made only in reporting of the samples' identities, and therefore the results were not excluded from the statistical analysis.

In the interlaboratory trial, the results obtained by each collaborating laboratory using the alternative method were exactly the same as those obtained by using the reference method. Consequently, the false-positive, false-negative and positive deviation values were all zero, and thus the alternative method was shown to be fully equivalent to the reference method.

In conclusion, the LAMP-based method of D'Agostino et al. (2015) for analysis of soya meal for *Salmonella* is fully equivalent to the corresponding international standard (Anonymous, 2002).

Acknowledgments We thank the Q-SAFFE project partners, particularly John Thompson and Sons Ltd., for the supply of feed samples. We thank Dr. Dario de Medici of ISS for assistance with the study.

Compliance with Ethical Standards

Funding The research leading to these results has received funding from the European Commission 7th Framework Project; Quality and Safety of Feeds and Food for Europe, QSAFFE (FP7-KBBE-2010-4, 265,702).

Conflict of Interest Avelino Alvarez-Ordonez has no conflict of interest.
Nigel Cook has no conflict of interest.
Martin D'Agostino has no conflict of interest.
Elisabetta Delibato has no conflict of interest.
Stephen Forsythe has no conflict of interest.
Patricia Gonzalez-Garcia has no conflict of interest.
Flemming Hansen has no conflict of interest.
John Ikonomopoulos has no conflict of interest.
Emily E. Jackson has no conflict of interest.
Kieran Jordan has no conflict of interest.
Petros Kokkinos has no conflict of interest.
Krzysztof Kwiatek has no conflict of interest.
Elżbieta Kukier has no conflict of interests.
Dubravka Milanov has no conflict of interest.
Vasileios Ntafis has no conflict of interest.
Lorna O'Brien has no conflict of interest.
Tamas Petrovic has no conflict of interest.
Susana Robles has no conflict of interest.
David Rodriguez Lazaro has no conflict of interest.
Zbigniew Sieradzki has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent Not applicable

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