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Validation on milk and sprouts of EN ISO 16654:2001 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Escherichia coli* O157

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ABSTRACT

In 2006, the European Committee for standardisation (CEN)/Technical Committee 275 - Food analysis - Horizontal methods/Working Group 6 - Microbiology of the food chain (TC275/WG6), launched the project of validating the method ISO 16654:2001 for the detection of *Escherichia coli* O157 in foodstuff by the evaluation of its performance, in terms of sensitivity and specificity, through collaborative studies. Previously, a validation study had been conducted to assess the performance of the Method No 164 developed by the Nordic Committee for Food Analysis (NMKL), which aims at detecting *E. coli* O157 in food as well, and is based on a procedure equivalent to that of the ISO 16654:2001 standard. Therefore, CEN established that the validation data obtained for the NMKL Method 164 could be exploited for the ISO 16654:2001 validation project, integrated with new data obtained through two additional interlaboratory studies on milk and sprouts, run in the framework of the CEN mandate No. M381. The ISO 16654:2001 validation project was led by the European Union Reference Laboratory for *Escherichia coli* including VTEC (EURL-VTEC), which organized the collaborative validation study on milk in 2012 with 15 participating laboratories and that on sprouts in 2014, with 14 participating laboratories. In both studies, a total of 24 samples were tested by each laboratory. Test materials were spiked with different concentration of *E. coli* O157 and the 24 samples corresponded to eight replicates of three levels of contamination: zero, low and high spiking level. The results submitted by the participating laboratories were analyzed to evaluate the sensitivity and specificity of the ISO 16654:2001 method when applied to milk and sprouts. The performance characteristics calculated on the data of the collaborative validation studies run under the CEN mandate No. M381 returned sensitivity and specificity of 100% and 94.4%, respectively for the milk study. As for sprouts matrix, the sensitivity resulted in 75.9% in the low level of contamination samples and 96.4% in samples spiked with high level of *E. coli* O157 and specificity was calculated as 99.1%.

1. Introduction

The ISO 16654:2001 is the international standard for the detection of *Escherichia coli* belonging to serogroup O157 in food and feed. The method is based on the possibility to capture *E. coli* O157 bacterial cells with magnetic beads coated with antibodies against the lipopolysaccharide antigen O157. It also exploits biochemical properties of *E. coli* O157 strains, such as the resistance to Cefixime Tellurite (CT) and the inability to ferment sorbitol, in order to facilitate their identification on a solid medium. Basically, ISO 16654:2001 describes the selective enrichment procedure of test samples in modified Tryptone Soya Broth (mTSB) supplemented with 20 mg/l Novobiocin, followed by the immunomagnetic separation (IMS) with beads coated with antibodies against O157 antigen and plating onto the differential and selective medium CT-Sorbitol MacConkey agar (SMAC) together with an alternative solid medium. *E. coli* O157 bacterial cells captured by magnetic beads and then streaked on CT-SMAC will appear as uncoloured colonies, due to the lack of sorbitol fermentation characteristic of most of *E. coli* O157 strains. The procedure includes also a confirmation step, to ascertain the isolated colonies as *E. coli* and by agglutinating them with

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an antiserum specific for O157.

The Nordic Committee for Food Analysis (NMKL) delivered another method, the NMKL method No 164, based on the same procedure (NMKL, 1999). The second edition of this method was issued in 2005 and included the data from a collaborative validation study carried out in 2002 by the National Food Administration, Uppsala, Sweden, which was conducted by challenging the method with three epidemiologically relevant food matrices (minced meat, raw milk and lettuce), contaminated with different concentrations of *E. coli* O157.

In 2006 the European Committee for standardisation or Comité Européen de Normalisation (CEN)/Technical Committee 275 - Food analysis - Horizontal methods/Working Group 6 - Microbiology of the food chain (TC275/WG6), launched the project of validating the ISO 16654:2001 by the evaluation of the method's performance through collaborative studies. In the same year, WG6 assessed the equivalence between the ISO 16654 and the NMKL method No 164, 2. Ed. 2005 and stated that the data from the collaborative validation study of the NMKL method No 164 could be used and transferred to the validation of the ISO 16654:2001 method. Therefore, the project concerning the validation of the ISO 16654:2001 under the CEN mandate M381 was based on the execution of a reduced validation study, upon agreement of CEN/TC275/WG6.

The European Union Reference Laboratory for *Escherichia coli*, including Verotoxigenic *E. coli* (EURL-VTEC) was assigned the leadership of the validation project and designed, organized and analyzed the results of the collaborative studies. In the project presented for the CEN mandate M381, only one epidemiologically relevant matrix, milk, was initially selected for the collaborative validation study, but in the aftermath of the huge outbreak of STEC infection linked to sprouts occurred in Germany in 2011, it was proposed by the EURL-VTEC, and agreed by CEN/TC275/WG6, to carry out an additional study on this particular matrix. In particular, the possibility to run the additional collaborative validation study on sprouts was explored and agreed in 2013 during a meeting organized by the EURL-VTEC with the laboratories that participated in the study on milk, and it was endorsed by CEN in October 2013.

The performance characteristics derived from the validation study of the NMKL method No 164 and the two collaborative studies run in the framework of the M381 mandate have been merged and included into the Amendment 1 of the ISO 16654:2001, which has been recently published (ISO, 2017).

The present paper reports on the results of the collaborative studies on milk and sprouts carried out under the CEN mandate M381 in 2012 and 2014.

2. Materials and methods

2.1. Design of the study

The method ISO 16654:2001 was challenged with two food matrices: raw milk and sprouts. Fifteen National Reference Laboratories (NRLs) from fourteen European Union (EU) Member States and one non-EU country participated in the collaborative study on milk, run in 2012. Fourteen laboratories (11 NRLs and three Official Laboratories) from 13 EU countries took part in the study on sprouts in 2014. All the participating laboratories were accredited in compliance with ISO/IEC 17025:2005 and had the ISO 16654:2001 in the list of accredited methods. Participants were enrolled through invitation by the EURL-VTEC, and at the time of the invitation the design of the study was also described. The participating laboratories were requested to apply the ISO 16654:2001 method, without any modification to the protocol described in the standard, to test the samples received in the framework of the collaborative validation studies. The results from all the participants were reported by compiling a form returned to EURL-VTEC, and the data were used to assess the performance characteristics of the method, as none of the laboratories was considered as outlier.

For each collaborative validation study, the food samples were contaminated with three different inoculum levels of *E. coli* O157 (zero, low and high inoculum). Participating laboratories received and analyzed 24 samples, which corresponded to eight blind replicates of each contamination level.

2.2. Preparation of test materials

The milk samples were prepared at Food Consumer and Product Safety Authority, NVWA, CHEK working group (Utrecht, NL), which carried out all the tests necessary to evaluate the stability and homogeneity of the samples. The characteristics of the test materials were reported in certificates, one for each level of contamination, provided by NVWA to the EURL-VTEC. The certificates included the information on the matrix, the contaminant, the spiking level, the uncertainty of measurement, date of preparation and expiry together with the code of the samples corresponding to the three different *inocula* of *E. coli* O157. Briefly, the milk samples contained naturally present background microflora and were contaminated with *Escherichia coli* O157 NCCB 100282, a strain that does not produce Shiga Toxins. The milk samples were supplemented with glycerol (11% final concentration) as cryopreservative, and eight blind replicates of each level of contamination were sent to each laboratory, for a total of 24 milk samples to be examined. The test portions consisted in 10 ml of milk and the samples had an expiry date set at one year if stored unopened at −70 °C or −80 °C. Test samples were prepared in May 2012, being the blanks prepared on the 11th and the contaminated samples on the 30th of May 2012. The samples were sent by NVWA to the laboratories in June 2012 as frozen specimens (in dry ice).

The samples for the collaborative study on sprouts were prepared at the EURL-VTEC. The matrix used in the collaborative validation study consisted of a commercial mixture of alpha-alpha (90%) and watercress (10%) sprouts acquired from a retailer.

Extensive testing of test materials was carried out in pre-trial studies on the same mixture of sprouts in order to ensure that the samples could be stable and homogeneous for the duration of the collaborative study. The fit-for-purpose of the samples was assessed on the base of achieving satisfactory results for stability and homogeneity.

The sprouts used as test material contained a natural background microflora that was evaluated by plating several dilutions of a sample of sprouts homogenized in physiological solution either on nutrient and selective agar plates, namely TSA and MacConkey agar. The use of the nutrient agar allowed the estimation of all background microflora, whereas on MacConkey agar it was possible to count *Enterobacteriaceae* only. The plate counts returned an estimation of 10^7 cfu/g on TSA and 10^5 cfu/g on MacConkey agar. Two randomly selected test portions, consisting of 25 g of non-spiked sprouts, were initially tested to assess the absence of *E. coli* O157 from the matrix used in the interlaboratory study.

The test strain used to contaminate the sprout samples was C210-03, a Shiga Toxin producing *E. coli* (STEC) O157 strain obtained from the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*, Statens Serum Institut (Copenhagen, DK) and included in the reference culture collection at EURL-VTEC. Strain C210-03 was grown on SMAC agar and a single colony was inoculated in TSB at 37 °C for 18 h. Spiking was carried out by adding to each sample 1 ml of conveniently diluted exponential culture of the test strain, grown in TSB. The uncertainty of measurement associated to the standardized inoculum, has been evaluated using the procedure described in the ISO/TS 19036:2006 (ISO, 2006). Test portions consisting of 25 g of sprouts were artificially contaminated on the 21st of March 2014 and the titer of the inoculum used for spiking was verified by plate count, by using SMAC agar plates. The samples were shipped as refrigerated on the 24th of March. The laboratories were requested to start the analysis upon receipt of the test materials, in order to carry out the analysis in the range of duration of stability, which had been previously

determined, as described further on.

2.3. Homogeneity and stability of test materials

Homogeneity and stability of test materials were assessed at Food Consumer and Product Safety Authority (NVWA) CHEK working group for milk samples, with the procedures in use at NVWA for sample preparation.

As for the sprout samples, the stability and homogeneity of the test materials were evaluated according to the requirements of ISO/IEC 17043:2010 (ISO, 2010).

2.4. Analysis of the data

The results of the studies on milk and sprouts were submitted to the EURL-VTEC by compiling a form provided to the participants, where it was requested to report the temperature of the samples upon receipt as well as the date of analysis.

The performance characteristics of the ISO 16654:2001 method determined were specificity, sensitivity and, when possible, the LOD_{50}. The sensitivity and specificity were calculated according to the following formulas:

$$\text{Sensitivity:SE} = [\text{true positives}/(\text{true positives} + \text{false negatives})] \times 100$$

$$\text{Specificity:SP} = [\text{true negatives}/(\text{true negatives} + \text{false positives})] \times 100.$$

The LOD_{50}, which is the concentration for which the probability of detection is 50%, was calculated according to Wilrich and Wilrich, 2009.

3. Results and discussion

3.1. Stability and homogeneity of the test samples

Homogeneity and stability of test materials were assessed at Food Consumer and Product Safety Authority (NVWA) CHEK working group for milk samples. In particular, homogeneity was assessed by testing with the ISO 16654:2001 standard a total of 10 samples of milk at each level of contamination. All the tests gave the expected results.

Stability of contaminated sprout samples had been previously assessed at EURL-VTEC on another batch of identical mixture of sprouts, during the preparation of a similar interlaboratory study (the Twelfth Proficiency Test, PT12, organized by the EURL-VTEC in 2013 for the benefit of the EU NRLs, report available at http://www.iss.it/binary/vtec/cont/PT12_Report.pdf) and samples resulted stable for at least eight days after contamination when stored refrigerated. All the tests were done on 26–27 March 2014, within the stability range.

The homogeneity of sprout test samples was evaluated by EURL-VTEC by testing two blank samples on the 19th–20th of March 2014, and 12 samples corresponding to the low- and high-levels of contamination on the 24th and 25th of March 2014 with ISO 16654:2001 method. As expected, blank samples resulted negative and high level contamination samples were found all positive for the presence of *E. coli* O157. Testing the low level of contamination allowed the assessment of fractional recovery, as out of 12 samples analyzed, only six samples with the low dose inoculum (10 cfu/g) were positive with the ISO 16654:2001 method, indicating that this analyte's concentration was around the limit of detection (LOD_{50}) for this method challenged with this specific matrix.

3.2. Interlaboratory study on milk

Milk samples were spiked at the three levels of contaminations, corresponding to: 0 cfu/ml, 25 cfu/ml (18–32 cfu/ml, according to the estimated combined uncertainty of measurement; 95% C.I.) and 140 cfu/ml (between 102 and 178 cfu/ml; 95% C.I.). Participating laboratories received eight replicates of each level of contamination. All the 15 participating laboratories received the test materials in the range of duration of the estimated stability and no labs reported to have applied deviations from the ISO 16654:2001 protocol. All the laboratories received the samples as frozen. The overall results of the collaborative study on milk submitted by the participating laboratories are shown in Table 1. More in detail, eleven laboratories (73,3%) submitted results concordant with the expected results, which were set as the gold standard. The remaining four labs provided a total of seven incorrect results, all false positive in blank samples (Table 1).

Table 1
Results of the collaborative validation study of ISO 16654:2001 on milk and sprouts. Number of concordant results/number of samples tested. In brackets Sensitivity (*Se*) for each contamination level and Specificity (*Sp*) evaluated for the blank samples are reported.

Milk			Sprouts		
Blank (0 cfu/ml)	Low level (25 cfu/ml)	High level (140 cfu/ml)	Blank (0 cfu/g)	Low level (10 cfu/g)	High level (100 cfu/g)
113/120 (*Sp* 94.4%)	120/120 (*Se* 100%)	120/120 (*Se* 100%)	111/112 (*Sp* 99.1%)	85/112 (*Se* 75.9%)	108/112 (*Se* 96.4%)

The data generated in this interlaboratory study were analyzed and sensitivity and specificity of the ISO 16654:2001 method in the conditions of this study resulted in 100% and 94.4%, respectively (Table 1).

3.3. Interlaboratory study on sprouts

The contamination of test materials was as follows: 0, 10 and 100 cfu/g of STEC O157, sent to the participating laboratories in eight blind replicates, for a total of 24 samples. The evaluation of the uncertainty of measurement associated with the spiking inoculum indicated that the concentration of STEC O157 ranged between 5 and 18 cfu/g (95% C.I.) for the low level of contamination and between 50 and 180 cfu/g (95% C.I.) for the high level of contamination. The fourteen laboratories received and examined the sprout specimens within the stability range. The temperatures upon receipt were in the range between +1 °C and +9 °C for the majority of the participating laboratories. Two labs reported to have received the samples as frozen and two didn't measure the temperature but reported that the cold plates in the parcel were still frozen.

An overview of the results reported by the participating laboratories in the study on sprouts, in terms of agreement of the results with the gold standard, is reported in Table 1. In particular, eight laboratories provided results concordant to those expected for all the 24 samples. The remaining six labs provided 32 results in disagreement with the expected results, and only one of these consisted in a false positive. The majority of the results in disagreement with the expected ones regarded the detection of *E. coli* O157 in sprout samples contaminated with the lowest concentration of the analyte. In particular, 27 out of the 32 non-concordant results concerned the lack of identification of *E. coli* O157 in the low level contamination samples (see Table 1). This was not surprising, as during the homogeneity tests the recovery fraction of the analyte was measured, and consisted in 50% of the samples spiked with 10 cfu/g (low level of contamination), indicating that this particular concentration was very close to the LOD_{50} of the method. The data obtained in the framework of the interlaboratory study were analyzed and allowed the identification of a more precise LOD_{50}, which resulted to be 8.4 cfu/g (6.5 cfu/g to 10.9 cfu/g confidence limit, 95% confidence interval). On the basis of the results obtained, sensitivity was 75.9% in the low level of contamination samples and 96.4% in samples spiked with high level of *E. coli* O157. Specificity was calculated as

being 99.1%.

4. Conclusions

The ISO 16654:2001 method has been applied for more than 15 years since its publication with excellent results, but proper data for validation were lacking. This gap was addressed by CEN/TC 275/WG6 issuing the mandate M381 supported by the European Commission, with the aim of validating a number of ISO standards, including ISO 16654:2001. The possibility to get benefit from the validation data obtained for the NMKL method No 164 on three epidemiologically relevant matrices such as milk, minced meat and lettuce, was exploited, as the two methods have been recognized as equivalent. This led to the decision to carry out two additional interlaboratory studies under the CEN mandate M381 on two food matrices only, milk and sprouts, particularly relevant as vehicles of STEC infection. The two studies conducted in the framework of the M381 mandate have been run in 2012 and 2014, and the results were comparable to those obtained in the framework of the validation studies conducted by National Food Administration, Uppsala, Sweden, for NMKL Method No 164. The lowest sensitivity values were calculated for the sprout samples, even if the concentration of the contaminating *E. coli* O157 was not the lowest used during the complete validation process, as in the interlaboratory studies conducted to validate NMKL method No 164 even lower concentrations had been examined. As a matter of fact, during the NMKL method No 164 validation study, the low-level contamination of minced meat and raw milk samples corresponded to 17 cfu/sample of *E. coli* O157, whereas for lettuce it was 10 cfu/sample versus the 250 cfu/samples used in the interlaboratory study on sprouts. In the validation study of NMKL method No 164, the sensitivity was assessed as 100% for lettuce and minced meat, and 92.8% for raw milk versus the 75.9% sensitivity assessed for sprouts for the low level contamination samples. These observations underline how much the matrix can affect when determining the performance of a method. Indeed the matrix sprout usually contains high levels of background microflora (Jinneman et al., 2012), in some cases estimated as 9×10^7 cfu/g by EURL-VTEC during pre-testing when organising a proficiency test on this matrix, which can hinder the detection of *E. coli* O157.

In the studies reported here, the milk samples were shipped in dry-ice as they contained glycerol as cryopreservative and no false negative results were recorded. The sprout samples were delivered as refrigerated, but two laboratories reported to have received them as frozen. One of the two laboratories correctly identified the presence/absence of *E. coli* O157 in all the test samples, whereas the other reported a few false negative results, either for the high- or low-levels of contamination. The attempt of explaining the false negative results is troublesome since the other laboratory reported all concordant results. A possibility could be that freezing may have induced stress in *E. coli* O157 bacterial cells, leading to difficulty in recovering the strain when applying the ISO 16654:2001. As a matter of fact, the viable but not culturable (VBNC) state is a phase in which bacteria undergo as a strategy in response to stressful conditions, remaining alive, but being unable to form colonies on media that normally support their growth. It has been recently observed that *E. coli* O157 cells may undergo the VBNC state after freezing (Liu et al., 2017), hindering the detection of the pathogen when using the cultural methods. It may be hypothesized that the false negative results obtained by one laboratory receiving the sprout samples as frozen could be explained by the presence of VNBC *E. coli* O157 cells in the samples.

Recently, Hara-Kudo and colleagues (Hara-Kudo et al., 2016) reported on an interlaboratory study aiming at the detection of STEC strains belonging to several serogroups in food matrices by Real Time PCR followed by isolation with IMS. In that study, radish sprouts were also tested for the presence of *E. coli* O157 at two different levels of contamination. The low level of contamination consisted in 4.8 cfu/25 g, much lower than the one applied for the present interlaboratory study on sprouts. In the Japanese study 83% of the low level contamination samples analyzed resulted positive for the presence of *E. coli* O157 following IMS, indicating that the LOD_{50} would be lower than the one estimated here. There are some differences between the two studies that need to be taken into account that could explain this discrepancy, such as the type of sprouts used. In fact, it has to be considered that sprouts obtained from seeds belonging to different species of vegetables may contain different background microflora or different substances, and it has been observed that naturally contaminating microflora may interfere with *E. coli* O157 and STEC growth, posing a challenge for their detection in different matrices (Duffy et al., 1999; Jinneman et al., 2012). The effect of the background microflora was the focus of a paper on the modelling of the growth of *E. coli* O157:H7 in ground beef, which showed that there is a simple competition between *E. coli* O157:H7 and the prevalent background microflora during the enrichment step, as it was observed that the *E. coli* O157:H7 growth halted as soon as the level of contaminating microflora reached the maximal value (Vimont et al., 2006).

Moreover, different number of replicates per level of contamination was analyzed (two in the case of the Japanese study versus the eight replicates for the validation of ISO 16654:2001). Altogether these considerations may account for the different results obtained in the two studies.

It is challenging to understand the reasons for the false positive results reported for the study on the milk samples: the possibility of cross-contamination during the analysis of a large number of samples, as the case of this interlaboratory study, exists and might have been a cause for these false positive results.

The results obtained in the two interlaboratory studies were presented to CEN/TC275/WG6 during the general meetings, and it was decided to include the performance characteristics of the method evaluated under the CEN mandate No. M381 together with those obtained in the validation study of NMKL method No 164 in an annex to the amendment of the ISO16654:2001. The amendment was drafted and subjected to voting at both CEN and ISO levels and, following approval, it was finally published in March 2017 as ISO 16654:2001/Amd.1:2017 (ISO, 2017).

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