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Validation by interlaboratory trials of EN ISO 10272 - Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 1: Detection method

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ABSTRACT

During the last decade *Campylobacter* has been the most commonly reported gastrointestinal bacterial infection in humans in the European Union. The use of a sensitive detection method based on enrichment of *Campylobacter* spp. is often needed when examining foods. However, as background flora developed resistance to third generation β-lactams used in selective culture media, the ISO method was adapted. It now consists of three different procedures (A, B, and C) depending on the expected concentration and condition of *Campylobacter* and the background microflora. As the diagnostic sensitivity of the detection test varies between laboratories, this justifies the validation of the method in an interlaboratory study.

The matrices selected for testing in the collaborative trials were frozen spinach (procedure A, Bolton enrichment broth), minced meat (procedure A, Bolton enrichment broth), raw milk (procedure B, Preston enrichment broth), chicken skin (procedure B, Preston enrichment broth), and broiler caecal material (procedure C, direct plating on mCCD agar). Each matrix was artificially inoculated with a different *Campylobacter* strain at a low and high contamination level, and with sterile diluent for 'blanks'. Seventeen laboratories participated in the interlaboratory study. The sensitivity and specificity of the methods for the five selected matrices were determined, as well as the level of detection (LOD_{50}). Calculated LOD_{50} values ranged from 0.84 cfu/test portion in frozen spinach and 2.2 cfu/test portion in minced meat to 14 cfu/test portion in chicken skin and 57 cfu/test portion in raw milk, all based on test portions of 10 g. The test portion size for broiler caecal material was a 10 µl-loop, yielding a LOD_{50} of 6.1 cfu/test portion. The validation data were incorporated in the newly published ISO standard EN ISO 10272-1:2017 - Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* - Part 1: Detection method.

1. Introduction

During the last decade *Campylobacter* has been the most commonly reported gastrointestinal bacterial infection in humans in the European Union (EFSA and ECDC, 2016). Broiler meat is considered the most common source of infection. Usually, with the exception of raw poultry meat, only low numbers of *Campylobacter* are present in foods. Often these bacteria are sub-lethally injured due to food preservation techniques or the influence of environmental factors and fail to grow on selective media after direct plating (Humphrey, 1989). A few hundred of *Campylobacter* cells can induce clinical gastrointestinal symptoms (Black et al., 1988). Therefore, the use of a sensitive detection method based on enrichment of *Campylobacter* spp. is often needed. A considerable number of different culture media and techniques for the isolation of *Campylobacter* from different matrices have been designed (Corry and Atabay, 2012). The International Standards Organization (ISO) standard method for the detection of *Campylobacter* spp., published in 2006, includes enrichment in Bolton broth and plating on mCCDA plus another selective agar medium; the media are incubated in a microaerobic atmosphere (Anonymous, 2006a). Every five years an ISO Standard is subject to Systematic Review in order to determine whether it should be confirmed, revised or withdrawn. During the 5-year review of ISO 10272-1:2006 the ISO Technical committee ISO/TC 34, *Food Products*, Subcommittee SC 9, *Microbiology* and the CEN

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Technical Committee CEN/TC 275 *Food analysis - Horizontal methods* concluded that the techniques described in this 2006-standard were not optimal for the detection of *Campylobacter* spp. in different matrices, which might result in an underestimation of the true prevalence of these bacteria. Consequently a technical revision of the standard was considered necessary, which was elaborated by the assigned expert members of CEN Task Advisory Group (TAG) 19-*Campylobacter*. The following main changes in the standard were applied:

1. As *Campylobacter* can be found along the total food chain, testing of samples from the primary production stage was added to the scope. Caecal content samples are commonly used to confirm *Campylobacter* colonization of broiler flocks.
2. The method was extended to include the option of a second enrichment broth (Preston broth), primarily to overcome problems with background flora resistant to third generation β-lactams (such as cefoperazone in Bolton broth). Although Bolton broth as an enrichment medium enables an effective resuscitation and growth of *Campylobacter*, this medium has a limited selectivity, which might inhibit the recovery of *Campylobacter* spp. from samples with a high level of indigenous flora, including ESBL producing *E. coli* (Jasson et al., 2009).
3. The method was extended to include the option of direct plating on mCCDA, in particular for broiler caecal content (Rodgers et al., 2012). Also, the overgrowth of competitive flora on mCCDA plates after enrichment might cause false negative confirmation of a positive sample. Therefore in case of samples containing a high level of background flora, such as chicken meat preparations, it was recommended to use an enrichment method and direct plating in parallel to obtain the best estimate of *Campylobacter* prevalence (Habib et al., 2008; Repérant et al., 2016).
4. The note on the use of closed containers with reduced headspace as an alternative to incubation in a microaerobic atmosphere was deleted. The volume of air in closed containers depends on the vessel chosen (permeability of e.g. a plastic bag or a glass or plastic bottle) and the depth of the headspace left by the operator. The metabolic activity of potential competitive flora (e.g. fermentative microbes consuming all the available oxygen) is another variable factor. This may lead to considerable differences between laboratories and the results obtained from closed vessels could differ significantly from enrichments incubated in a microaerobic atmosphere (Moran et al., 2009).
5. The confirmation tests on the study of microaerobic growth at 25 °C and aerobic growth at 41.5 °C were replaced by the study of aerobic growth at 25 °C as the former two growth tests sometimes give results that are difficult to interpret.
6. Performance testing for the quality assurance of the culture media according to ISO 11133 (Anonymous, 2014) has been added to the standard.

The revision and validation of the first edition of ISO 10272-1 (Anonymous, 2006a), resulted in a second version of this standard (Anonymous, 2017a) in which also the performance characteristics, established as described in this paper, have been included.

In the field of food hygiene, including production environments, there is a lack of standardised and validated methods. Investigation of the culture method results, used to estimate the prevalence of *Campylobacter*-contaminated broiler carcasses in a European baseline survey, showed that the diagnostic sensitivity of the detection test varied between the participating laboratories (EFSA, 2010).

Within the framework of the European food hygiene legislation, the European Union (EU) aims at availability of standardised validated methods that are referred to in legislation in order to support the EU food policy. Commission Regulation No (EC) 2073/2005 on microbiological criteria for foodstuffs contains provisions requiring the use of standard analytical methods in the food chain (Anonymous, 2005). In this Regulation a reference method has been specified for each microbiological criterion. Horizontal EN or EN ISO methods, when available, have received the status of reference methods in the Regulation and therefore should be standardised and validated. The validation of European standards is also of importance since it will lead to improved recognition of the standards by the international community, improved substantiation of the microbiological criteria and therefore improved consumer protection, and a better recognition of analytical results by courts, the European Food Safety Authority, other authorities and laboratory inspections at international level.

The European Union, represented by the European Commission, decided to award a grant for the elaboration of 15 published European Validated Standards (EN) in the field of food hygiene legislation under mandate SA/CEN/ENTR/381/2010-06 “Microbiology of Food and animals stuffs” (Mandate M/381) (Anonymous, 2010). The agreements were established by the European Commission and the European Committee for Standardization (CEN). The ‘Deutches Institut für Normung e. V’ (DIN) and the ‘Association Française de Normalisation (AFNOR)’ participated as members of CEN in this specific grant agreement. The elaboration of the standards was performed by subcontractors under the mandate M/381, in which The Netherlands Food and Consumer Product Safety Authority (NVWA, Wageningen/Utrecht, the Netherlands) was the appointed subcontractor to perform validation work on the standard for detection of *Campylobacter* described in ISO 10272-1 (Anonymous, 2006a, 2017a) and the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) was the appointed subcontractor to perform validation work on the standard for enumeration of *Campylobacter* described in ISO 10272-2 (Anonymous, 2006b, 2017b). The subcontractors were responsible for the collaborative validation studies, including preparation of protocols, data collection and evaluation. The institutes closely collaborated on the elaboration of the interlaboratory studies to validate both parts of ISO 10272.

The aim of the study described in this manuscript was the validation of the three procedures for detection of *Campylobacter* as described in the revised standard ISO 10272-1 (Anonymous, 2017a), for sample types that are usually tested for the prevalence of *Campylobacter*. The detection of *Campylobacter* can be performed using three different procedures, depending on the expected levels of *Campylobacter* and background flora;

- procedure A: Detection of *Campylobacter* by selective enrichment in Bolton broth, in samples with low numbers of *Campylobacter* and a low level of background microflora and/or with stressed *Campylobacter*, e.g. cooked or frozen products;
- procedure B: Detection of *Campylobacter* by selective enrichment in Preston broth, in samples with low numbers of *Campylobacter* and a high level of background microflora, e.g. raw meats (including poultry) or raw milk;
- procedure C: Detection of *Campylobacter* by direct plating, in samples with high numbers of *Campylobacter*, e.g. faeces, poultry caecal contents or raw poultry meat.

In general, detection procedure B is useful for products (including cooked or frozen) that contain significant numbers of microflora resistant to third generation β-lactams like cefoperazone. Cefoperazone is used in Bolton broth (detection procedure A) as well as in mCCD agar. Preston broth (detection procedure B) uses different selective agents and is therefore more suitable to suppress this type of resistant microflora.

In this manuscript we report the results of the collaborative studies performed in October/November 2013 and the results of the statistical analysis to determine the specificity, sensitivity, and level of detection at 50% (LOD_{50}) of the qualitative methods.

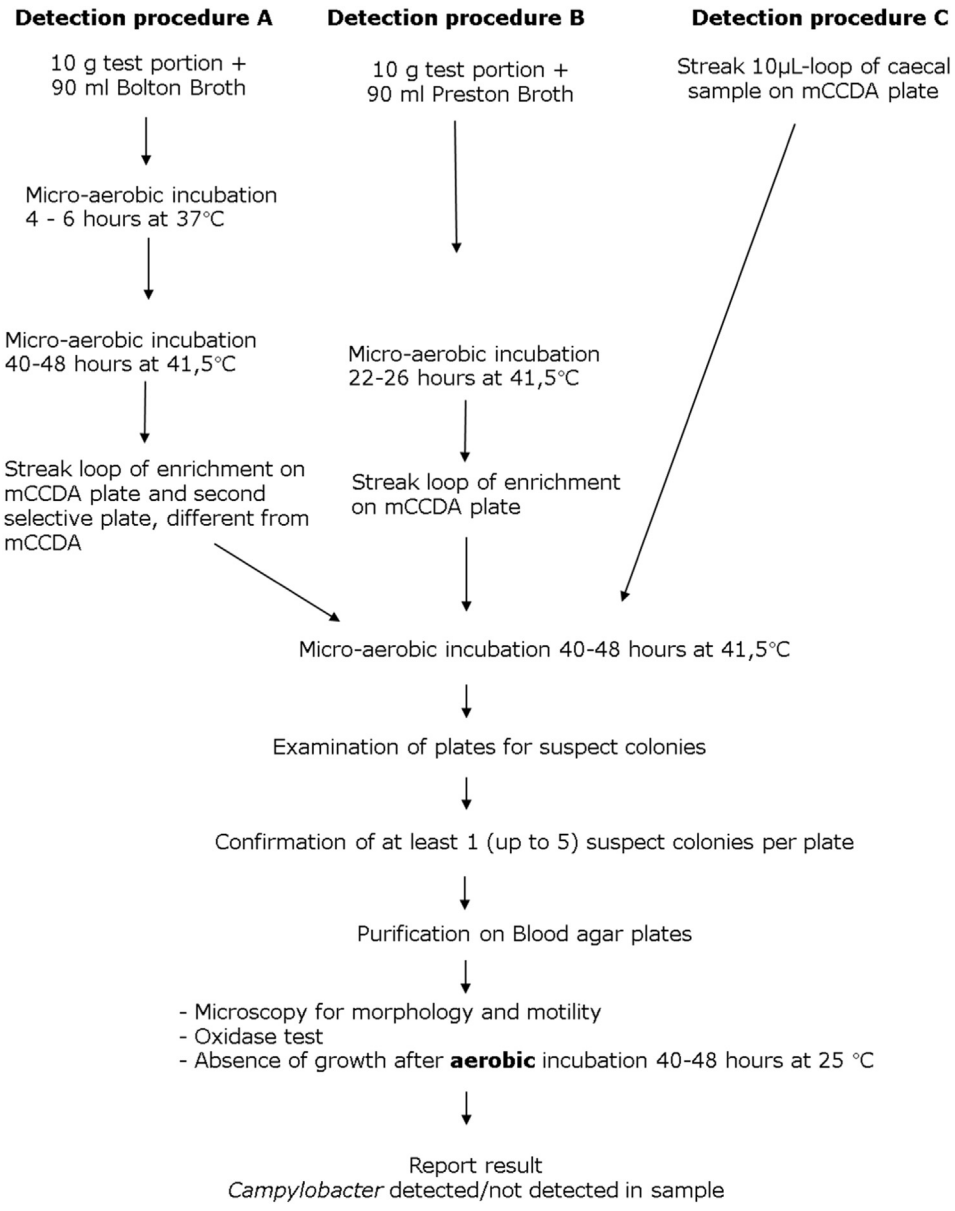

Fig. 1. Diagram of the procedures for the detection method as used in the interlaboratory trial.

2. Materials and methods

2.1. Design of the Trial

The design of the trial was basically according to the description of the interlaboratory study for qualitative methods in ISO 16140:2003 (Anonymous, 2003). Five different matrices were selected to be tested in the collaborative trials, taking into account the three different detection procedures (A, B, C) in order to validate the method horizontally. The selected matrices represent sample types that are usually tested for the prevalence of *Campylobacter* (Jacobs-Reitsma et al., 2008) and concerned frozen spinach (procedure A), minced meat (procedure A), raw milk (procedure B), chicken skin (procedure B), and broiler caecal material (procedure C). Each matrix was artificially inoculated with a different *Campylobacter* strain: *C. jejuni* WDCM 00005 (frozen spinach), *C. coli* WDCM 00072 (minced meat), *C. jejuni* WDCM 00156 (raw milk), *C. coli* WDCM 00004 (chicken skin) and *C. jejuni* DSM 24306/CNET 076 (broiler caecal material). These strains are recommended for the quality control of culture media according to ISO 11133 (Anonymous, 2014) and according to the Handbook of Culture Media for Food and Water Microbiology (Corry and Atabay, 2012). Three levels of contamination per matrix were tested: blank, low (close to the detection limit of the method), and high (approximately 10 times the detection limit of the method). *Campylobacter*-negative broiler caecal material was artificially contaminated with *Campylobacter* at RIVM, prior to shipment of the samples. Due to the vulnerable nature of the micro-organism, *Campylobacter* may not stay stable when distributing inoculated food samples. Therefore the other matrices had to be artificially contaminated by the participants after receiving the samples, using the provided vials with pre-prepared *Campylobacter* cultures. This approach has been successfully used in previous validation studies (Rosenquist et al., 2007) and is also commonly used by the European Union Reference Laboratory (EURL) for *Campylobacter* in their ringtrials within the network of National Reference Laboratories (NRLs) (Ingrid Hansson, EURL-*Campylobacter*, personal communication). Eight blind replicates were used per level and matrix, resulting in 120 samples to test per laboratory. All test materials during the study were randomly coded in order to prevent biased analysis based on expected outcomes.

According to ISO 16140:2003 (Anonymous, 2003) a minimum of 10

valid data sets (80 results per level and per matrix) are needed for qualitative studies, but some more than just 10 participating laboratories would be preferred in order to be able to compensate for any unforeseen exclusions of data.

The members of TAG 19-*Campylobacter* of CEN/TC275/WG6 "Microbiology of the food chain", as well as the network of EU NRLs-*Campylobacter* were contacted for participation in the study. Participants were informed about the time schedule and procedure of the validation process prior to the start of the study. Each participating laboratory was randomly assigned a unique participant code. Participants were provided with a Standard Operating Procedure (SOP), containing the detailed description of the culture methods to be used, a procedure for artificially inoculating the samples prior to investigation, a form to report the condition of the samples upon receipt and a form to report the materials used, incubation conditions and times, and final results (also see the SOP provided as a Supplementary Data File). The test materials (matrix samples plus inoculation vials) were shipped under UN3373 conditions to the participants on 28 October 2013, using dry ice in polystyrene boxes and a courier service. All media and materials needed for analysis of the samples were provided by the participants. The participants had to start the analysis using procedures B and C on Monday November 4, 2013 and the analysis using procedure A on Monday November 18, 2013. Results were to be reported by December 11 the latest. The provided data were statistically analyzed as described in Section 2.4.

2.2. Method for validation

The method was validated for test portions of 10 g for the matrices frozen spinach (procedure A), minced meat (procedure A), raw milk (procedure B) and chicken skin (procedure B), and 0.01 ml (using a 10 µl loop) for the matrix broiler caecal material (procedure C).

The diagram of the procedures for the detection method as used in the interlaboratory study is shown in Fig. 1. On the day of analysis the 10 g test portions had to be defrosted in upright position for 45 min at room temperature and then stored in the refrigerator until actual start of the testing. The vials with preinoculated caecal material and the vials with *Campylobacter* inoculation suspensions had to be defrosted for 30 min at room temperature. A specified amount from each inoculation vial had to be added to the corresponding sample bag, followed by the addition of the appropriate selective enrichment broth and homogenization by using a Stomacher at medium speed for 1 min (also see the SOP provided as a Supplementary Data File).

The microaerobic atmosphere for incubation of *Campylobacter* is described to have an oxygen content of 5% ± 2%, carbon dioxide 10% ± 3%, optional hydrogen ≤10%, with the balance nitrogen. Participants were asked to use their customary microaerobic atmosphere and record this on the result form.

2.3. Preparation and stability testing of the test materials

Five different matrices were used for the collaborative trials. Fresh *Campylobacter*-free chicken carcasses for chicken skin sample preparations were imported from Sweden (Guldfågeln AB, Mörbylånga, Sweden). *Campylobacter*-negative caecal material was kindly provided by Albert ter Laak (Wageningen Bioveterinary Research, Lelystad, The Netherlands). One batch of frozen spinach and one batch of minced meat (beef/pork) were obtained from a local supermarket. Raw cow's milk was directly obtained from a dairy farm near Zutphen (The Netherlands). Ten samples per matrix were tested for the absence of *Campylobacter* using ISO 10272-1:2006 before processing. All test portions were prepared at RIVM and the non-inoculated matrix samples were stored at −20 °C before dispatching.

The caecal material was artificially contaminated with strain *C. jejuni* DSM 24306/CNET 076 at RIVM and stored at −80 °C before dispatching. The other matrices had to be artificially contaminated by the participants just before starting their analysis, using a dedicated vial per sample with (or without) *Campylobacter* cultures. All vials for inoculations were prepared by CHEK (nowadays: Biosisto, Assen, the Netherlands). Overnight cultures of the strains (*C. jejuni* WDCM 00005, *C. coli* WDCM 00072, *C. jejuni* WDCM 00156, *C. coli* WDCM 00004) were prepared at the desired concentrations in blood-based medium with glycerol and stored at −80 °C before dispatching. Participants were instructed on the inoculation of the samples in the Standard Operating Procedure (SOP) for the collaborative study (see Supplementary Data File).

Pre-tests, in series of 8 samples for each matrix and inoculation level, were done at RIVM and NVWA to check on the suitability of the matrix-inoculation vials combinations, to obtain the desired levels of contamination of the various sample types. It was intended to have a "fractional recovery" (preferably about 50% of the samples to be found positive and 50% to be found negative) at the low level inoculation samples, in order to be able to calculate the LOD_{50}. The high level was approximately 10 times the low level. All pre-tests started at a low inoculum level of around 5 cfu/sample. Results from the first test series indicated e.g. that this level was too high for the matrix spinach (all samples positive), but too low for the matrix raw milk (all samples negative) to obtain a fractional recovery per 8 samples tested. Further series of samples were tested, gradually adjusting the inoculum level per matrix to determine the appropriate inoculum to result in fractional recovery.

The homogeneity of the samples was determined according ISO guide 34 (Anonymous, 2009). The homogeneity was determined for each batch of the matrix/inoculation vials combinations by determining the level of *Campylobacter* on mCCDA and on non-selective blood agar for 10 vials. If the obtained value was below the critical value, the batch of vials was considered homogeneous.

The stability during storage at −80 °C was determined by analyzing the various batches of vials at different time intervals after production, as described for homogeneity. To determine the homogeneity and stability of the vials the between-groups variation and the within-groups variation was calculated, using ANOVA. If the obtained value was below the critical value, the batch of vials was considered homogeneous and stable.

The actual *Campylobacter* concentration of the vials on the day of use for inoculation of the samples was determined on non-selective blood agar plates by NVWA and RIVM, analyzing 4 vials each per matrix/level combination.

2.4. Analysis of the data

Data have been excluded from the calculations only on the basis of clearly identified technical reasons (temperature or time abuse during transport of the samples or deviations from the Standard Operating Procedure by participants).

The sensitivity, the specificity, and the level of detection at 50% (LOD_{50}) were determined as the performance characteristics of the detection method. The sensitivity was defined as the number of samples found positive divided by the number of true positive samples tested at a given level of contamination. The sensitivity results were thus dependent on the level of contamination of the sample. The specificity was defined as the number of samples found negative divided by the number of blank samples tested (Anonymous, 2017a). The LOD_{50} is the concentration in cfu per sample for which the probability of detection is 50%. The calculations for LOD_{50} were performed using a dedicated Excel sheet (Wilrich and Wilrich, 2009).

3. Results

3.1. Homogeneity and stability of the test materials

The homogeneity and stability of the vials for artificial inoculation

Table 1
Number of *Campylobacter*-positive confirmed results reported by the participants for eight replicates per matrix and per contamination level.

Matrix	Inoculum	Laboratory code																
	Level[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Frozen spinach	Blank	0	0	0	0	**2**	0	0	0	0	0	0	0	–	**1**	0	0	0
	Low	**4**	**7**	**6**	**5**	8	**5**	**7**	**5**	8	**7**	**6**	**5**	–	**5**	**6**	**7**	**3**
	High	8	8	8	8	8	8	8	8	8	8	8	8	–	8	8	8	8
Minced meat	Blank	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0
	Low	**4**	**7**	**6**	**2**	**0**	**5**	**1**	**2**	**6**	**2**	**4**	**2**	–	**2**	**5**	**4**	**4**
	High	8	8	8	8	**4**	8	8	**5**	8	**7**	8	8	–	8	8	8	**5**
Raw milk	Blank	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0
	Low	8	8	8	**0**	**5**	8	**0**	**0**	**3**	**0**	8	**0**	–	**3**	8	8	**1**
	High	8	8	8	**3**	**6**	8	**2**	**0**	**7**	**4**	8	8	–	**7**	8	8	**7**
Chicken skin	Blank	0	0	0	0	0	0	0	–	0	0	0	0	–	**1**	0	0	0
	Low	**7**	**7**	8	**3**	**4**	8	8	–	8	**3**	**3**	**4**	–	**3**	**6**	**5**	**0**
	High	8	8	8	8	8	8	8	–	8	8	**6**	**7**	–	**5**	8	8	**4**
Caecal material	Blank	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0
	Low	**1**	**2**	**0**	**2**	**0**	**3**	**3**	**1**	**2**	**5**	**1**	**1**	**5**	**5**	**5**	**2**	**0**
	High	8	8	**7**	8	8	8	8	8	8	8	8	8	8	8	8	8	**7**

–: Excluded from the dataset due to deviations to the protocol.
In bold: deviations from the expected 0 positives (Blanks) or from the maximum expected 8 positives (Low, High).
[a] Actual inoculation levels in cfu per test portion are given in Table 2.

was tested and all batches were found to be homogeneous and stable. The inoculum levels per matrix, as calculated from the enumerations on the vial contents by NVWA and RIVM, are indicated in Table 2. No problems were reported regarding the transport and receipt of the samples. All participants started the analysis within the given timeframe.

3.2. Results of the interlaboratory studies

Seventeen laboratories from 13 different countries in Europe participated in the interlaboratory study. Table 1 shows the overview of the data as reported by the participants.

Laboratory 13 was excluded from the dataset for the matrices frozen spinach, minced meat, raw milk, and chicken skin, since the inoculation process of the matrices was not successful and therefore no *Campylobacter* could be detected in any of the matrices at any contamination level. This same laboratory was able to detect *Campylobacter* in the matrix broiler caecal material which was send to all laboratories preinoculated, indicating that this laboratory was able to detect *Campylobacter* and there was no problem with the method itself. Laboratory 8 was excluded from the dataset for the matrix chicken skin, due to a reported operator error for this matrix.

Most laboratories incubated in jars, creating a microaerobic atmosphere either using sachets ($n = 6$) or flushing with an appropriate gas mixture ($n = 6$), or both ($n = 1$). Four laboratories used microaerobic incubators. The analysis protocol was respected by all laboratories.

3.3. Performance assessment of the method

The method was validated for test portions of 10 g or ml, except for the matrix broiler caecal material which was validated for a test portion of 0.01 ml (10 µl inoculation loop).

The specificity for all matrices is included in Table 2. For all matrices tested the specificity was above 95%.The sensitivity for all matrices, per inoculation level, is also included in Table 2. LOD_{50} calculations require a fractional recovery at the low level as a minimum. Therefore, the inoculum levels of the matrices were aiming at this and as a consequence resulted in a low sensitivity of the methods at the low inoculum levels. The LOD_{50} values could be calculated for all matrices (fractional recovery for at least the low level) and are displayed in Table 2. Calculated LOD_{50} values ranged from 0.84 cfu/test portion in frozen spinach and 2.2 cfu/test portion in minced meat to 14 cfu/test portion in chicken skin and 57 cfu/test portion in raw milk, all based on test portions of 10 g. The test portion size for broiler caecal material was a 10 µl-loop, yielding a LOD_{50} of 6.1 cfu/test portion.

4. Discussion

The sensitivity and specificity for the five selected matrices were determined, as well as the LOD_{50}. As a result from three false positive blank samples, the specificity of the method for the different matrices ranged from 98 to 100%. These false positives could have been caused by a natural *Campylobacter* contamination in these matrices, though this seems to be very unlikely as all pre-tests on the matrices as well as all other blank tests did not detect any *Campylobacter*. More likely these findings would have been due to cross contamination in the laboratory or errors in the initial sample inoculation procedure. The sensitivity was influenced by the type of matrix, the contamination level and the *Campylobacter* strain (Hazeleger et al., 2015). In addition, the frozen condition of the samples before and during transport of the samples may have influenced the sensitivity due to changes in background flora when compared to fresh samples. To obtain a fractional recovery as required for the LOD_{50} calculations, the inoculum levels of the matrices were low which resulted in a low sensitivity of the method at low inoculum levels. For high levels of contamination the sensitivity of the method ranged from 78 to 100% depending on the type of matrix tested. For low levels of contamination this range was 28–64%. In an interlaboratory study on a Nordic method for the detection of *Campylobacter* in food an overall specificity of 98.6% and an overall sensitivity of 82.8% was found for the qualitative detection of *Campylobacter* (Rosenquist et al., 2007). As the validation protocol used in the Nordic study differs in many details from the protocol used in our study a proper comparison of the results is not possible. The sensitivity results obtained in our study for raw milk were poor compared to the other matrices. A possible explanation is the presence of a lactoperoxidase system in raw milk, which might inhibit the growth of *Campylobacter* (Beumer et al., 1988). Being correlated to the sensitivity, the LOD_{50} value is also matrix and strain dependent and ranged from 0.84 cfu per test portion of frozen spinach to the rather high 57 cfu per test portion of raw milk. When implementing this *Campylobacter* detection method, the user laboratory may want to verify this by determining the in-house LOD_{50} value and compare this to the value reported in the ISO method. The detection method was validated for test portions of 10 g or ml, except for the matrix broiler caecal material which was validated for a

Table 2
Summary of the performance characteristics for the detection method for the matrices frozen spinach, minced meat, raw milk, chicken skin, and broiler caecal material.

Matrix	Frozen spinach			Minced meat			Raw milk		
Detection procedure	Procedure A (Bolton broth)			Procedure A (Bolton broth)			Procedure B (Preston broth)		
Strain used for inoculation	*C. jejuni* WDCM 00005			*C. coli* WDCM 00072			*C. jejuni* WDCM 00156		
Level of inoculation	Blank	Low	High	Blank	Low	High	Blank	Low	High
Enumeration of the inoculation vials (n = 8) in Mean log10 cfu/ml ± SD	–	1.5 ± 0.1	2.5 ± 0.1	–	1.5 ± 0.1	2.0 ± 0.1	–	1.3 ± 0.5	2.5 ± 0.3
Level of inoculation in cfu/test portion	–	1.6	16	–	1.3	9.6	–	17	206
Number of participating collaborators	17	17	17	17	17	17	17	17	17
Number of samples per collaborator	8	8	8	8	8	8	8	8	8
Number of collaborators retained after evaluation of the data	16	16	16	16	16	16	16	16	16
Number of sample results retained after evaluation of the data	128	128	128	128	128	128	128	128	128
Test portion size in gram	10	10	10	10	10	10	10	10	10
Sensitivity in %	–	73	100	–	45	91	–	53	78
Specificity in %	98	–	–	100	–	–	100	–	–
LOD_{50} (95% confidence interval) in cfu/test portion	–	0.84 (0.67 to 1.0)		–	2.2 (1.8 to 2.6)		–	57 (46 to 70)	

Matrix	Chicken skin			Caecal material		
Detection procedure	Procedure B (Preston broth)			Procedure C (direct plating)		
Strain used for inoculation	*C. coli* WDCM 00004			*C. jejuni* DSM 24306		
Level of inoculation	Blank	Low	High	Blank	Low	High
Enumeration of the inoculation vials (n = 8) in Mean log10 cfu/ml ± SD	–	1.4 ± 0.3	2.6 ± 0.2	–	1.4 ± 0.2	3.8 ± 0.0
Level of inoculation in cfu/test portion	–	7.5	105	–	0.55	136
Number of participating collaborators	17	17	17	17	17	17
Number of samples per collaborator	8	8	8	8	8	8
Number of collaborators retained after evaluation of the data	16	16	16	16	16	16
Number of sample results retained after evaluation of the data	128	128	128	128	128	128
Test portion size in gram	10	10	10	0.01	0.01	0.01
Sensitivity in %	–	64	92	–	28	99
Specificity in %	99	–	–	100	–	–
LOD_{50} (95% confidence interval) in cfu/test portion	–	14 (11 to 19)		–	6.1 (3.1 to 12)	

test portion of 0.01 ml (10 µl inoculation loop). As a general rule and therefore mentioned in all recently revised and validated ISO standards, a smaller test portion than validated may be used, without the need for additional validation/verification, providing that the same ratio between enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no adverse effects on the detection.

From the results of the validation study it can be concluded that the methods described in the revised standard EN ISO 10272-1:2017 are satisfactory for the detection of *Campylobacter* in different matrices. However, the values derived from this interlaboratory study may not be applicable to food types or strains other than those that were tested in this study (Hazeleger et al., 2015).

The choice of the test protocol is based on the expected number of *Campylobacter* and the level of background microflora. It will not always be easy for laboratories to select the most appropriate method when little information is available concerning the particular type of sample to be tested. In these cases it is recommended to use different culture procedures in parallel to obtain the best estimate of the prevalence of *Campylobacter* spp. (Habib et al., 2008). The fact that different enrichment media may select for particular species of *Campylobacter* (Williams et al., 2012) also advocates the parallel use of different enrichment and isolation protocols.

It will be important to consider laboratory results obtained in routine testing of samples of different matrices, using protocols described in EN ISO 10272-1:2017 during the next Systematic Review of this standard. Regarding the culture media, mCCD agar is generally considered to be the best choice as the first isolation medium to be used (Rodgers et al., 2012; Gharst et al., 2013). The use of a second isolation medium with different selective and diagnostic properties is still considered necessary for the inhibition of growth of competing microflora after enrichment. Some of the recently introduced novel chromogenic agars can be an effective addition to the use of mCCDA. Modifications of Bolton broth to include potassium clavulanate and triclosan have been suggested to prevent the growth of ESBL *E. coli* (Seliwiorstowa et al., 2016). During the revision of ISO 10272-1:2006 it was decided to maintain the addition of blood to the enrichment media. Most of the enrichment media for the detection of *Campylobacter* include blood to protect the organisms from the toxic effects of oxygen. In some studies, however, no significant differences were observed in performance of enrichment broth supplemented with blood and without blood (Odongo et al., 2009; Gharst et al., 2013). During the revision it was also decided to keep the change in temperature from 37 °C to 41.5 °C after the first 4–6 h of incubation in Bolton broth (procedure A). But also this detail may be re-considered at the next Systematic Review, as recent work (Hazeleger et al., 2016) has shown that this more troublesome part of procedure A hardly has any beneficial effect.

5. Conclusion

The EN ISO 10272-1 method for detection of *Campylobacter* was validated for the matrices frozen spinach, minced meat, raw milk, chicken skin and broiler caecal material. The sensitivity and specificity for the five selected matrices were determined, as well as the LOD_{50}. Calculated LOD_{50} values ranged from 0.84 cfu/test portion in frozen spinach and 2.2 cfu/test portion in minced meat to 14 cfu/test portion in chicken skin and 57 cfu/test portion in raw milk, all based on test portions of 10 g. The test portion size for broiler caecal material was a 10 µl-loop, yielding a LOD_{50} of 6.1 cfu/test portion. The validation data were incorporated in the newly published ISO standard EN ISO 10272-1:2017 - Microbiology of the food chain - Horizontal method for detection and enumeration of *Campylobacter* - Part 1: Detection method (Anonymous, 2017a).

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Appendix A. Supplementary data

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