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Abstract: EN ISO 10273 method for the detection of pathogenic Yersinia enterocolitica in foods was validated in the project Mandate M/381 funded by European Commission. A total of 14 laboratories from five European countries participated in the interlaboratory study (ILS) organized during 2013 and 2014. Before the ILS, the method was revised by an international group of experts and the performance of the revised method was assessed in an ILS study. The results are published as a part of the standard EN ISO 10273 revision. The study included three rounds with different sample types; raw milk, iceberg lettuce and minced meat, inoculated with a low and high level of pathogenic Y. enterocolitica strains representing major pathogenic bioserotypes 4/O:3 and 2/O:9. The homogeneity and stability of the samples were verified before dispatching them to the laboratories. The results demonstrated the method sensitivity of 96% in raw milk, 97% in minced meat, and 98% in lettuce at high inoculation level of pathogenic Y. enterocolitica. The specificity was 100% in raw milk, 96 % in minced meat, and 98% in lettuce. The level of detection, LOD50, varied between study rounds, being 9.4 CFU/25 ml in raw milk, 9.9 CFU/25 g in minced meat and 63 CFU/25 g in lettuce samples. During the study, confirmation by using real-time PCR method ISO/TS 18867 together with pyrazinamidase testing was also validated, as alternative to conventional biochemical confirmation. When comparing different isolation steps used in the revised method during the study rounds, PSB enrichment and plating on CIN after alkaline (KOH) treatment showed the highest sensitivity (52-92%) in raw milk and minced meat samples. In lettuce samples, however, ITC with KOH treatment before plating on CIN showed higher sensitivity (64% at low level; 82% at high level) than plating on CIN from PSB with KOH treatment (44% at low level; 74% at high level). Statistical analysis of different isolation steps supported the use of two enrichment media, PSB and ITC, in the revised method. Recovery of pathogenic Y. enterocolitica on CIN was most efficient after KOH treatment and, based on the analysis, plating on CIN agar without KOH treatment could be left as optional procedure in the method.

Validation of ISO method 10273 - Detection of pathogenic *Yersinia enterocolitica* in foods

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Keywords: validation, pathogenic Y. enterocolitica, detection, standardization, interlaboratory study

Abstract

EN ISO 10273 method for the detection of pathogenic *Yersinia enterocolitica* in foods was validated in the project Mandate M/381 funded by European Commission. A total of 14 laboratories from five European countries participated in the interlaboratory study (ILS) organized during 2013 and 2014. Before the ILS, the method was revised by an international group of experts and the performance of the revised method was assessed in an ILS study. The results are published as a part of the standard EN ISO 10273 revision. The study included three rounds with different sample types; raw milk, iceberg lettuce and minced meat, inoculated with a low and high level of pathogenic *Y. enterocolitica* strains representing major pathogenic bioserotypes 4/O:3 and 2/O:9. The homogeneity and stability of the samples were verified before dispatching them to the laboratories. The results demonstrated the method sensitivity of 96% in raw milk, 97% in minced meat, and 98% in lettuce at high inoculation level of pathogenic *Y. enterocolitica*. The specificity was 100% in raw milk, 96 % in minced meat, and 98% in lettuce. The level of detection, LOD_{50}, varied between study rounds, being 9.4 CFU/25 ml in raw milk, 9.9 CFU/25 g in minced meat and 63 CFU/25 g in lettuce samples. During the study, confirmation by using real-time PCR method ISO/TS 18867 together with pyrazinamidase testing was also validated, as alternative to conventional biochemical confirmation. When comparing different isolation steps used in the revised method during the study rounds, PSB enrichment and plating on CIN after alkaline (KOH) treatment showed the highest sensitivity (52–92%) in raw milk and minced meat samples. In lettuce samples, however, ITC with KOH treatment before plating on CIN showed higher sensitivity (64% at low level; 82% at high level) than plating on CIN from PSB with KOH treatment (44% at low level; 74% at high level). Statistical analysis of different isolation steps supported the use of two enrichment media, PSB and ITC, in the revised method. Recovery of pathogenic *Y. enterocolitica* on CIN was most efficient after KOH treatment and, based on the analysis, plating on CIN agar without KOH treatment could be left as optional procedure in the method.

1 Introduction

The project to elaborate and validate a series of 15 European Standards (EN) in the field of food hygiene legislation was initiated following the agreement signed between the European commission and the European Committee for Standardization (CEN) in December 2010, in response to Mandate M/381. At that time, the standard EN ISO 10273 for the detection of presumptive pathogenic *Yersinia enterocolitica* in

foods was last updated in 2003 and lacked collaboratively tested performance criteria. The enrichment and plating steps within the method had been selected on the basis of historical user experience, but not subjected to thorough international collaborative testing.

For the revision and validation of ISO 10273, as part of the Mandate M/381 project, a team of *Y. enterocolitica* methodology experts evaluated and revised the method based on the most recent literature and knowledge of the *Y. enterocolitica* detection methodology. Before revision, the isolation method included enrichment of a test portion in peptone, sorbitol and bile salts (PSB) broth as a tenfold dilution, followed by plating onto Cefsulodin, IrgasanTM and Novobiocin (CIN) agar after potassium hydroxide (KOH) treatment and without KOH treatment. In parallel, the method included enrichment of a test portion in Irgasan^{TM}, ticarcillin and potassium chlorate (ITC) broth as a 100-fold dilution, followed by plating onto Salmonella-Shigella-desoxycholate-calcium chloride (SSDC) agar without KOH treatment (Anonymous 2003).

During the revision, the method was complemented with direct plating on CIN agar, and incubation times were shortened for PSB broth (from 5–6 days to 44 ± 4 h) and CIN agar (from 48 h to 24 ± 2 h), both of which had been recommended previously (EFSA 2009; Van Damme et al., 2010). In the revised method, 90 ml of ITC was inoculated with 10 ml of initial suspension in PSB, and SSDC agar was replaced with CIN agar and an optional chromogenic medium. Previous studies on slaughter pig tonsils samples revealed no significant difference between *Y. enterocolitica* counts on CIN and SSDC media (Van Damme et al., 2010). The performance of chromogenic *Y. enterocolitica* media for qualitative and quantitative isolation of pathogenic *Y. enterocolitica*, however, was equal to SSDC and CIN (Van Damme et al. 2012). Furthermore, direct plating on CIN, or KOH treatment after ITC enrichment, was recommended to replace the conventional ITC-SSDC step (Van Damme et al., 2012), and thus, KOH treatment after ITC enrichment was included in the revised method.

For the confirmation of pathogenic *Y. enterocolitica*, tests related to pathogenicity were specified and added in the frontline in the revised method. Determination of pathogenic *Yersinia* (genus level) involves four tests, while confirmation of pathogenic *Y. enterocolitica* (species level) involves seven tests (conventional biochemical testing) in the revised method. Therefore, it is more convenient to first identify pathogenic *Yersinia* isolates for further confirmation. Additionally, as the tests related to pathogenicity were made mandatory and allowed the separation of pathogenic from non-pathogenic *Y. enterocolitica*, the word “presumptive” could be replaced with the word ”pathogenic” in the standard title.

After the revision, the method was subjected to an interlaboratory study from May 2013 until April 2014, and the results were summarised in EN ISO 10273:2017 (Anonymous, 2017). The aim of the study was to determine sensitivity, specificity, and the level of detection (LOD_{50}) of the method. Based on the confirmed

results of all isolation steps of the method, the necessity of parallel enrichment steps and alkaline treatment in the method were also evaluated. In confirmation of pathogenic *Y. enterocolitica*, the performance of real-time PCR ISO/TS 18867 (Anonymous 2015), together with pyrazinamidase testing, was assessed as an alternative to conventional biochemical confirmation. Furthermore, the performance testing scheme for the quality assurance of the culture media used in the method was verified. We report here the results of the interlaboratory study to validate EN ISO 10273.

2 Materials and methods

2.1 Design of the trial

A total of 14 laboratories from five European countries (Belgium, Finland, Germany, Norway and Sweden) participated in the interlaboratory study (ILS) organized in 2013 and 2014. The laboratories were experienced in the analysis of pathogenic *Y. enterocolitica*, 11 of the laboratories were accredited according to EN ISO 17025 and three laboratories were established in scientific research of pathogenic *Y. enterocolitica*. The design of the study followed the document ISO/TC34/SC9 N 851 (CEN/TC275/WG6 N 323) "Basis of the mandate study' and included three rounds with different sample matrices; raw milk, minced meat, and green crispy lettuce. Before the ILS, method comparison studies were performed by the organizing laboratory (Finnish Food Safety Authority Evira) during 2011–2013. Furthermore, a pre-trial was organized with 14 participants before the ILS to allow the laboratories to become acquainted with the laboratory procedure and to verify the suitability of the test material for the purpose.

Samples were artificially inoculated to ensure pre-defined inoculation levels and the required homogeneity of samples. For each food type, two inoculum levels; low and high, and blank samples were used based on the preliminary evaluation of the level of detection (LOD_{50}) (for inoculum CFU) for each matrix by the organizing laboratory. During each trial round, each participant laboratory analysed 24 randomly coded samples, i.e. 8 replicates at each inoculation level. To ensure that the samples were handled similarly, the laboratories were instructed to start the analyses the day after dispatch, at the earliest (between approximately 18 and 24 h after dispatch). The laboratories were allowed to start analysing all 24 samples at the same time or in two sets on instructed starting days. Both sets contained an equal number of samples from each level (blank, low and high). The samples were shipped in insulated EPS packages (Biotherm 45 DI 96, DGP Intelsius Ltd. UK) in dry ice and qualified to maintain integrity for 96 h. A temperature data logger (LOG-IC USB Multi Use Logger with Ribbon Probe, American Thermal Instruments, Ohio) was inserted in each package for monitoring of the sample temperature during shipping. In addition, laboratories were asked to record the temperature near the sample tubes when unpacking the samples. The laboratories performed the analyses according to detailed instructions (laboratory procedure) provided

by the organizer and reported the results on a separate form, including information on factors which may have affected their results (exact incubation times, culture media batches and preparation used, etc.). The protocol used during the ILS study served as the basis for the standard, to which no major technical changes were made before publication.

2.2 Method used in the interlaboratory study

The ILS study protocol consisted of the following successive stages for the detection of pathogenic *Y. enterocolitica*:

a) Pre-treatment of food samples according to standardized procedure to obtain initial suspension and inoculation of the suspension (direct plating) onto 2 to 4 selective agar plates (CIN).
b) Enrichment in liquid enrichment medium (PSB) and selective liquid enrichment medium (ITC) at 25 °C for 44 h.
c) Plating out the enrichment with KOH treatment (mixing 0.5 ml of enrichment in 4.5 ml of 0.5% KOH solution for 20 s), and without KOH treatment on CIN agar, followed by incubation at 30 °C for 24 h and verification of the colony morphology as presumptive pathogenic *Y. enterocolitica* by successive culturing on selective plates.
d) Confirmation of the presence of pathogenic *Y. enterocolitica* by appropriate biochemical or molecular confirmation tests. The determination of pathogenic *Yersinia* species was performed first to ease the workload in the following confirmation of pathogenic *Y. enterocolitica*.

In the interlaboratory study, confirmation by using alternative pathway (see Figure S1 in supplementary material) was validated against the conventional biochemical confirmation pathway of ISO 10273. As the alternative pathway, collaborators used real-time PCR for detecting pathogenic *Y. enterocolitica* as described in ISO/TS 18867 (Anonymous 2015), Annex B, Methods 1 or 2 and pyrazinamidase testing.

According to the standard, it is not necessary to confirm from all successive enrichment steps if pathogenic *Y. enterocolitica* from an earlier step was already confirmed. However, during the interlaboratory study, the laboratories agreed to confirm all the steps separately to obtain results for the evaluation of necessity of each step in the method.

Laboratories were allowed to use commercially available dehydrated culture media, ready-to-use culture media or preparation from separate ingredients. For enrichment and plating media, majority of the laboratories (10/14) prepared PSB from separate ingredients, and four laboratories used dehydrated medium Peptone Sorbitol Bile Broth (Fluka / Sigma-Aldrich). The majority of the laboratories (12/14) used dehydrated ITC broth base and respective supplements from various manufacturers (Fluka / Sigma-Aldrich,

ten laboratories; HiMedia, two laboratories; Merck, one laboratory). Two laboratories prepared ITC from separate ingredients. For CIN agar, nine laboratories used dehydrated agar base and supplements (Oxoid, seven laboratories; Merck, four laboratories; LabM, three laboratories). Four laboratories used ready-to-use CIN agar (Oxoid, three laboratories; Tammer-Tutkan maljat Oy, two laboratories).

In addition, laboratories had the opportunity to voluntarily use chromogenic media in parallel to CIN plating. YECA agar (bioMérieux, Marcy l'Etoile, France) (Denis et al. 2011) was used by ten laboratories during the study round with minced meat, and CHROMagar™ *Y. enterocolitica* (CHROMagar, Paris, France) (Renaud et al. 2013) was used by seven laboratories during the study rounds with raw milk, minced meat, and lettuce. In each ILS round, one enrichment was decided for the plating onto chromogenic agars beforehand to reduce the workload.

During the ILS study, three laboratories tested cold enrichment procedure as described in EN ISO 10273 Annex D (Anonymous, 2017). The cold enrichment procedure included:

a) Direct culture on CIN, sample enrichment in PSB or CEB (cold enrichment broth) at 4 °C for altogether 22 ± 1 days.
b) From the enrichment, after 8 ± 1 and 22 ± 1 d of incubation, inoculation of MRB broth (modified Rappaport broth with magnesium chloride), incubation at 25 °C for 4 d and plating on CIN.
c) From the enrichment, after 14 ± 1 and 22 ± 1 d of incubation, plating on CIN with and without KOH treatment.
d) Incubation of CIN plates at 30 °C for 24 h. Identification of typical colonies and confirmation according to EN ISO 10273 (see 2.2; c and d).

2.3 Preparation of the test materials

The food matrix in the first ILS round in May 2013 was unpasteurized (raw) cow's milk, collected from the tank of Helsinki University farm, Finland. The matrix for the second ILS round in November 2013 was atmosphere-packed minced meat (beef-pork, 50-50%) originating from Finland. In the third ILS round in February 2014, green crispy lettuce originating from Finland was used. All matrices were divided into portions of 25 ml or 25 g, and stored at -70°C until use. Intrinsic background microbiota of the matrices was analysed by colony count at 30°C (EN ISO 4833:2013) and by enumeration of *Enterobacteriaceae* (EN ISO 21528-2:2004). The mean aerobic plate counts were 1.1 x 10^3 CFU/ml for milk, 9.8 x 10^3 for minced meat, and 1.1 x 10^5 CFU/g for lettuce. The mean *Enterobacteriaceae* counts were <10 CFU/ml for milk, 10 CFU/g for minced meat, and 4.2 x 10^3 CFU/g for lettuce. The absence of pathogenic *Y. enterocolitica* in all matrices was confirmed by real-time PCR ISO/TS 18867 targeting the *ail*-gene (Thisted Lambertz et al. 2008, Anonymous 2015).

The strain used for the inoculation of raw milk samples was *Y. enterocolitica* bioserotype 4/O:3 (Evira 595), isolated from pork at Evira, Finland, in 2010. For minced meat samples, *Y. enterocolitica* bioserotype 2/O:9 (Evira 663) strain isolated from a pig carcass swab in Belgium, 2011 was used by the courtesy of Prof. Lieven De Zutter, Ghent University, Belgium. Lettuce samples were inoculated with *Y. enterocolitica* bioserotype 2/O:9 strain (Evira 589), isolated from human faeces during a suspected carrot-borne outbreak in Finland, 2010, used by the courtesy of Prof. Anja Siitonen, National Institute for Health and Welfare, Finland. All the strains used for inoculation were confirmed to contain the *ail*-gene by real-time PCR (Thisted Lambertz et al. 2008) and virulence plasmid pYV by CR-MOX test (Riley and Toma 1989). The strains represented the pathogenic bioserotypes of *Y. enterocolitica* (4/O:3 and 2/O:9) that are the most prevalent in many parts of the world (Bottone, 1999).

For preparation of the inoculum, each ILS strain (Evira 595, 663 and 589) was streaked onto blood agar and incubated overnight at 30°C. One colony was transferred into TSB broth (BBL™ Trypticase™ Soy Broth, BD™, Thermo Fisher Scientific, Becton, Dickinson and Company, 38800 Le Point de Claix, France) and incubated at 30°C for 24 h ± 3 h. The incubated broth culture was serially diluted and the last dilution was made in 20% skim milk solution. Aliquots of skim milk suspension were stored at -70°C until used for inoculation. From the same aliquots of skim milk, three parallel platings onto blood agar were performed to determine the concentration of the bacteria in the aliquots at the time of freezing.

For the inoculation of the samples, the skim milk aliquots were thawed at room temperature for 30 min. Subsequently, 100 µl, containing the defined number of bacteria for low and high contamination level (see Table 1), were added onto each frozen sample. Inoculation at low level was designed to facilitate the calculation of the level of detection (LOD_{50}) by returning fractional positive results (not every sample positive or negative). For the determination of the number of inoculated bacteria, 100 µl aliquots of skim milk suspension were plated onto four parallel blood agar plates at regular intervals during the inoculation of samples (8 to 11 times, resulting in a total of 32 to 44 plates on each level). After the inoculation, the samples were immediately transferred back to -70 °C for storage until the time of dispatch. All plates were incubated for 44 ± 4 h at 30 °C, and the number of inoculated bacteria at each level was calculated as the mean value of all the counts obtained. The blank samples were prepared separately (on a different day) from inoculated samples by adding sterile skim milk on frozen samples. The samples were sealed tightly and handled separately until packing for transportation with other samples.

2.4 Homogeneity and stability of the test materials

For homogeneity studies, analyses of 60 samples (20 samples from each contamination level: blank, low and high) were started 13 (raw milk study), 6 (minced meat study) or 8 days (lettuce study) before the start of each ILS round. The analyses for homogeneity were conducted according to the ILS laboratory procedures. In addition, the homogeneity of the prepared inoculum was assessed from the colony counts determined when inoculating the samples (see 2.3).

For stability studies, 60 samples of each round (20 samples from each level: blank, low and high) were divided into two sets of 30 samples which were stored under two different conditions representing optimal transport conditions (Stability study 1, 10 samples from each level) and suboptimal transport and storage conditions (Stability study 2, 10 samples from each level). To mimic optimal transport conditions, the samples were packed in dry ice and the transport packages were kept at room temperature (22 to 25 °C) for 20 to 24 h before the start of the analysis. To mimic suboptimal transport conditions, the samples were packed in dry ice and the transport packages were kept at room temperature for three days, after which the packages were opened and the samples were transferred for storage at -20 °C for three or four days before the start of the analysis.

Additionally, the stability of the strain suspension that was used for inoculation was verified towards successive thawing and re-freezing by colony count (as described in 2.3) before each trial round. This was to ensure that the number of bacteria remained stable during the additional freezing in the inoculation process (see 2.3).

2.5 Statistical analysis of the data

The plate counts of inocula for homogeneity assessment were analysed according to ISO/TS 22117:2010 Annex B, $T_1 - T_2$ test and acceptable variation between units of a batch were considered to be $T2 / (I - 1) \leq 2$.

The LOD_{50} was calculated using PODLOD calculation program version 5 (Wilrich and Wilrich, 2009, ISO 16140-2:2015, http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html, accessed 14.3.2017)

A logistic regression model (*M1*) was applied to evaluate the necessity of parallel enrichment steps and alkaline (KOH) treatment in the laboratory procedure. The effects of ten complementary options for the method (*T2*–*T11*, Table 4), later referred to as treatments, on the sensitivity of detection were compared with relation to the reference; direct plating of PSB initial suspension on CIN agar (without enrichment) (*T1*). The variation of detection that arose from the experimental setting was considered in the model by including fixed effects for the contamination level and ILS, and random effect for the laboratories.

$$\text{logit}\ (\theta_i) = \mu_0 + \alpha_1 \cdot T1_i + \alpha_2 \cdot T2_i + \alpha_3 \cdot T3_i + \alpha_4 \cdot T4_i + \alpha_5 \cdot T5_i + \beta[Level_i] + \gamma[ILS_i] + \delta[Lab_i] \quad (M1)$$

$$y_i \mid \theta_i \sim Bin\ (n_i, \theta_i),$$

in which y_i is the number of true positive samples detected per contamination level per ILS per laboratory

n_i is the number of true positive samples per contamination level per ILS per laboratory

θ_i is the proportion of true positive samples detected (or sensitivity) per contamination level per ILS per laboratory

with uninformative prior probability densities

$$\begin{aligned}
&p\ (\mu_0) = N(0, 10^3) \\
&p\ (\alpha_j) = N(0, 10^3), \quad j = 1,..., 5 \\
&p\ (\beta_k) = N(0, 10^3), \quad k = 1, 2 \\
&p\ (\gamma_l) = N(0, 10^3), \quad l = 1, 2, 3 \\
&p\ (\delta_m) = N(0, \tau^{-1}), \quad m = 1,..., 14 \\
&p\ (\tau) = Gamma(10^{-3}, 10^{-3})
\end{aligned}$$

Detection probabilities and odds ratios (ORs, relative to *T1*) for the single treatments *T2–T5* were defined with equations (*E1*) and (*E2*). These were further used for calculating detection probabilities and odds ratios for the combined treatments *T6–T11* with equations (*E3*) and (*E4*).

$$\text{logit}\ (p(Tj)) = \mu_0 + \alpha_j, \quad j \in \{1,..., 5\}, \quad \alpha_1 = 0 \quad \text{for the reference} \quad T1 \quad (E1)$$

$$\text{OR}(Tj) = e^{\alpha_j}, \qquad j \in \{2,..., 5\} \quad (E2)$$

$$p(TB) = 1 - \prod_{r=1}^{R} [1 - p(TA_r)], \quad TB \in \{T6,..., T11\}, \quad TA_r \in \{T1,..., T5\} \quad (E3)$$

$$\text{OR}(TB) = \frac{p(TB)/(1 - p(TB))}{p(T1)/(1 - p(T1))}, \quad TB \in \{T6,..., T11\} \quad (E4)$$

Data were analysed by using the R software (version 3.3.2; R Core Team [https://www.R-project.org/]) and OpenBUGS software (version 3.2.3 rev 1012; OpenBUGS Project Management Group

[http://www.openbugs.net/]) via R2OpenBUGS package. Dataset contained only the observations from treatments *T1–T5* on low and high contamination level, and observations with missing values were removed before the analysis. Markov Chain Monte Carlo simulations were run in one chain with 10 000 iterations after adaptation of 2,000 iterations. For each OR, mean and 95% posterior credibility interval were calculated from 10 000 iterations. If 95% interval for OR > 1.0, treatment was interpreted to increase the sensitivity of detection with relation to the reference. Differences in effects between two treatments were reported if their 95% intervals for ORs appeared non-overlapping. In addition to the treatment effects over all ILS rounds, datasets were formed and analysed for each ILS separately to observe any variation between food matrices or strains. After evaluating the results, analyses were repeated by setting *T2* (PSB enrichment with KOH) as the reference. Datasets and codes for R and OpenBUGS are available from the authors upon request.

3 Results and discussion

3.1 Homogeneity and stability of the test materials

In homogeneity testing, the inoculated raw milk, minced meat and lettuce samples gave the expected numbers of positive results. Furthermore, the plate counts determined during preparation of the inocula were within the acceptable variation at the batch level in each study round (Table S1). The mean counts for low and high inoculum level were respectively 9 CFU (standard deviation, SD 3.0 CFU) and 59 CFU (SD 7.7 CFU) for raw milk; 16 CFU (SD 4.0 CFU) and 85 CFU (SD 15 CFU) for minced meat; and 110 CFU (SD 8.6 CFU) and 1 100 CFU (SD 11 CFU) for lettuce. Thus, the samples and the inocula were considered homogenous. Likewise, with expected number of positive results, the samples were considered stable, even under suboptimal shipping conditions (Table S1).

3.2 General results of the study

The majority of the laboratories received their samples within 27 h after dispatch and could directly proceed to analysis or storage of the samples. Exceptionally, three laboratories in the study round with minced meat and one laboratory in the study round with lettuce received their samples two days after dispatch. They stored the samples at -20°C or -70°C and started the analysis seven days after dispatch. As indicated by the temperature logger data and individual temperature measurements by the laboratories, the temperature was stable during shipping. Moreover, the samples were verified to be stable for altogether seven days (see 3.1).

After evaluation of deviations, results of two laboratories in the study round with raw milk were excluded from further analysis based on deviation from the protocol. For one laboratory in the study round with lettuce, one blank sample and one sample with the low inoculation level were excluded from the analysis due to lack of confirmation tests. The results of the other laboratories could be included after detailed examination and requests of further information, to ensure that there were no deviations from the protocol.

Summary of participant laboratories' positive sample results at each inoculation level and sample type is given in Table 1.

3.3 Analysis of the data

3.3.1 Specificity

The laboratories examined eight blank samples per food type, a total of 24 samples expected to give negative results. In ILS round with raw milk, no false positive results were observed. In ILS round with minced meat, three laboratories reported false positive results (Table 1); one laboratory with two out of eight samples and two laboratories with one sample out of eight. Six isolates recovered by two laboratories from a blank sample were sent to the organizing laboratory for further analysis. In ILS round with lettuce, two laboratories reported one false positive result each. The isolates recovered were available for further characterization from one of the laboratories. All the isolates were indistinguishable from the strains used for inoculation of the samples, based on the results obtained from biochemical tests and biotyping according to ISO 10273, *ail*-gene detection by ISO/TS 18867 and PFGE analysis (data not shown). This suggests cross-contamination of the samples, either by the organizer or by the participating laboratories, but the site of the cross-contamination could not be verified. Every precaution was taken at the organizing laboratory not to cross-contaminate the samples. Neither did the participants report leakage of the samples during shipment. The specificity of the method was 100% with raw milk (Table 2), 96% with minced meat (Table 3) and 98% with lettuce samples (Table 4).

3.3.2 Sensitivity

The overall sensitivity of the method at the high contamination level was 96% with raw milk (Table 2), 97% with minced meat (Table 3) and 98% with lettuce samples (Table 4). Based on the study design, a sensitivity around 50% was expected for the low level. The sensitivities at the low inoculation level were 68% for raw milk, 77% for minced meat, and 81% for lettuce.

Participating laboratories agreed to confirm separately all the parallel steps of the method leading to isolation (see 2.2 for the explanation of the procedure), which made it possible to compare the necessity of parallel steps for the general outcome of the procedure. Participant laboratories' positive results and samples analysed at each enrichment and plating step of the method are summarized in Table 5.

The sensitivity of direct culture varied from 2% to 21% in raw milk and minced meat with the low and high inoculation level and in lettuce matrix with the low inoculation level, whereas the sensitivity increased to 64% in lettuce samples with the high inoculation level (Table 5). Despite of this surprisingly high recovery of pathogenic *Y. enterocolitica* in lettuce matrix without any enrichment, direct culture alone was insufficient for optimal recovery in all three matrices. However, direct culture was added in the revised method as mandatory due to its capacity to give rapid results in certain cases of food sample analysis.

The sensitivity of PSB enrichment step in different sample types varied between 44% and 90% when KOH treatment was used before plating, and between 3% and 24% in the absence of KOH treatment. When using KOH treatment before plating, PSB was the most sensitive enrichment step throughout the study except for lettuce samples, where enrichment in ITC and KOH treatment before plating on CIN showed the highest sensitivity 82% (high inoculation level). In comparison, the sensitivity of PSB and KOH was 74% (high inoculation level) in lettuce. In raw milk and minced meat samples, the sensitivity of PSB with KOH treatment varied between 52% and 90%. The sensitivity of ITC varied between 15% and 82% with KOH treatment before plating, and between 7% and 60% without KOH treatment, in all three matrices.

3.3.3 Statistical analysis for the effect of isolation steps on sensitivity

Of the single treatments (*T2*–*T5*), the highest OR (95% interval: 9.4–15.3) was observed for *T2* (PSB with KOH) with relation to the reference *T1* (direct culture) in the analysis over all ILS rounds. Furthermore, treatment *T2* increased the sensitivity of detection more than *T4* (ITC with KOH; OR 3.3–5.2), and thus, higher performance was observed for PSB than for ITC enrichment. For both PSB and ITC, higher performance was observed with KOH treatment than without it. Without KOH treatment, ORs appeared respectively 0.3–0.5 (*T3*) and 1.2–2.0 (*T5*). This suggests that the isolation step without KOH was unnecessary and could be omitted from the method.

Because the highest performance was observed for *T2* with relation to the reference *T1*, treatment *T2* was set as the new reference to further evaluate the necessity of parallel steps in the method. In the analysis over all ILS rounds, treatment *T8* (parallel enrichment in PSB and ITC, both with KOH treatment) was

observed to increase the sensitivity of detection (OR 1.6–2.0) with relation to the reference *T2* (PSB with KOH) (Table 6). Odds ratios of similar order were observed for *T10* and *T11*, respectively 1.9–2.4 and 2.3–3.3, suggesting that the sensitivity of detection cannot be increased significantly by adding direct culture and steps without KOH in the method. However, direct culture was agreed to be a mandatory part of the procedure already during the revision because of its advantage to produce rapid results with certain sample types. Therefore, *T10* was chosen as the mandatory procedure in the method, containing direct culture in addition to parallel enrichment in PSB and ITC, plating both with KOH treatment.

When ILS rounds were analysed separately, the performance of the enrichment broths were observed to vary between the food matrices (or strains). In the ILS rounds for raw milk and minced meat, lower performance was observed for ITC (*T4*) compared with the reference, PSB (*T2*). In contrast, higher performance (OR 1.4–3.0) was observed for ITC (*T4*) in the ILS round for lettuce. This further supports parallel inclusion of PSB and ITC in the standard method. Apparently, ITC successfully suppressed the background microbiota of lettuce matrix and therefore compensated the performance of non-selective PSB in detection of pathogenic *Y. enterocolitica*. Omitting steps without KOH from the method was also supported by the separate analyses of the ILS rounds for minced meat and lettuce, which provided no evidence on the difference in performance between steps with KOH treatment (*T10*) and when supplemented with steps without KOH (*T11*). For raw milk, however, the inclusion of step without KOH was beneficial: slightly higher OR (2.4–3.9) was observed for *T11* than for *T10* (1.6–2.3). As the data revealed, three laboratories detected positive samples at low level (one, two, and three samples positive out of eight, respectively) from PSB without KOH that remained negative with other treatments. This supports the optional inclusion of step without KOH in the method, which can be advantageous with certain sample types.

Noteworthy, the better performance of KOH treatment depends on the success of the treatment. This is mostly affected by the preparation and shelf life of KOH, specified as “preparation the day before use” in the standard revision. The better performance of PSB compared to ITC, in turn, could be explained by the inoculum size; the initial suspension of ITC is prepared by transferring 10 ml of initial suspension in PSB into 90 ml of ITC and thus resulting in smaller fraction of the bacteria in enrichment than with PSB. For certainty of transferring at least one microorganism (*Yersinia* cell, in this case) in 1 ml, the suspension requires average level of at least seven cells per ml of the culture (Jarvis, 2007). Thus, the lower recovery when using ITC compared with PSB enrichment may have been purely by chance of not transferring any of the inoculated cells into ITC at the low inoculation level. This could have affected isolation especially from milk samples, in which the inoculum was the lowest. Along with fewer cells transferred to ITC, selective ingredients of ITC could inhibit also the growth of *Y. enterocolitica*, resulting in PSB to surpass ITC.

Especially, this could affect isolation from sample matrices in which the selective properties of ITC are dispensable or isolation of strains that are more susceptible. De Zutter et al., 1994 reported that growth of serotype O:9 in ITC was supressed in comparison to serotype O:3 in pork samples, especially due to potassium chlorate of ITC.

3.3.4 Level of detection LOD_{50}

The low inoculation level included in the ILS was designed to give fractional positive results making it possible to assess LOD_{50} of the method, meaning the specific concentration of pathogenic *Y. enterocolitica* that gives 50% probability of detection. Values of LOD_{50} varied depending on the combination of the matrix and strain studied. In raw milk inoculated with *Y. enterocolitica* bioserotype 4/O:3 strain, LOD_{50} was 9.4 CFU/25 ml. In minced meat and lettuce, each inoculated with different *Y. enterocolitica* bioserotype 2/O:9 strain, LOD_{50} was 9.9 CFU/25 g and 63 CFU/25 g, respectively (Tables 2-4). The relatively higher LOD_{50} in lettuce may have resulted from the presence of higher background microbiota, such as *Enterobacteriaceae*, which may have adverse effect on the level of detection. Because the enrichment and plating media used in the method are not highly selective for pathogenic *Y. enterocolitica*, other members of *Enterobacteriaceae* family, non-pathogenic *Yersinia* and even non-pathogenic environmental *Y. enterocolitica* can easily overgrow pathogenic *Y. enterocolitica.* Furthermore, these organisms may be picked for confirmation instead of pathogenic *Y. enterocolitica* if the laboratory is not experienced with the analysis.

However, preliminary studies at the organizing laboratory showed that LOD_{50} varied notably depending on the strain used for inoculating the lettuce matrix (data not shown).This suggests that the pathogenic *Y. enterocolitica* strain has a major role in the probability of detection, besides the background microbiota and the composition of food. Eventually, no single isolation procedure appears to be absolute for the recovery of all human-pathogenic *Y. enterocolitica* strains in foods (De Boer, 2003; Fredriksson-Ahomaa and Korkeala 2003).

The effects on LOD_{50} using different enrichment and plating steps and the combinations of them are shown in Table 7. With direct culture as the only step in the method, LOD_{50} values were approximately ten times higher as compared to the whole protocol. The whole protocol consisted of direct plating on CIN agar, parallel enrichment in PSB and ITC, both followed by parallel plating onto CIN with and without KOH treatment. By performing only the direct plating and PSB with KOH, LOD_{50} values approached those achieved by performing the whole protocol with raw milk and minced meat, but not with lettuce. With lettuce samples, LOD_{50} remained approximately ten times higher unless a combination of PSB and ITC was used. Steps that were selected as mandatory in the revised EN ISO 10273 resulted in LOD_{50} values of similar

order (12 CFU/25 ml in raw milk, 11 CFU/25 g in minced meat and 68 CFU/25 g in lettuce) when compared with the whole protocol. Mandatory steps of the revised standard differed from the whole protocol by exclusion of the plating without KOH step after PSB and ITC enrichment.

3.3.5 Use of chromogenic media

Only five laboratories included chromogenic media in their test procedure in all three ILS rounds. To increase the comparability of the results, evaluation was based on seven laboratories that used chromogenic media in both rounds with minced meat and lettuce. In each round, one enrichment for the plating onto chromogenic agars was decided beforehand. The results are summarized in Table S2 where comparison to the respective (parallel) CIN plating step can be made. Choosing the enrichment broth beforehand aimed to reduce the workload of laboratories but did not always produce the maximum number of presumptive positive colonies for confirmation. For example, in ILS round with minced meat, ITC was chosen beforehand as enrichment for the plating onto chromogenic agars, but PSB appeared to be more sensitive enrichment in that round. The sensitivity of chromogenic media was usually lower than or equal to CIN (Table S2). However, the sensitivity was equal or superior to CIN in the low and high inoculation level lettuce samples, respectively, when plated from PSB with KOH treatment. Overall, the limited number of results showed high variation. For example, one laboratory reported 16 positives out of 16 samples and found the chromogenic agar beneficial, while three laboratories reported no positives and other three laboratories reported one to three samples positive from the corresponding minced meat samples (data not shown). The usefulness of chromogenic media to the overall sensitivity of detection seemed to be limited. Only three laboratories reported single correct positive results by using chromogenic agars while parallel CIN agar plates remained negative, in minced meat and lettuce samples. The results for chromogenic agars are only indicative and should be interpreted with caution. The study resulted not enough data to justify the mandatory use of chromogenic media as primary plating media after enrichment, but supported its optional use in the method.

3.3.6 Confirmation of the results in ISO 10273

In the revised standard method ISO 10273 (Anonymous 2017), suspected colonies from primary culture are streaked (purified) on CIN agar and (optionally) on chromogenic agar to facilitate selection of characteristic colonies for further confirmation. The selection based on colony morphology (Figure S2) is done by using a stereomicroscope which is emphasized in the revised standard method. Microscoping can markedly reduce the number of isolates needing further confirmation and thus decrease the workload. During the ILS, chromogenic media performed especially well in distinguishing between colonies of potentially pathogenic

and non-pathogenic *Yersinia* when choosing isolates for confirmation. The same can also be achieved by using CIN (Hallanvuo et al., 2006) but this requires more expertise on colony morphology than simple colour difference detection in chromogenic media. However, when intrinsic sample microbiota in ILS study contained non-pathogenic *Y. enterocolitica* –like species *Y. mollaretii* and/or *Y. bercovieri*, their colonies could not be differentiated from pathogenic *Y. enterocolitica* based on colour on chromogenic media. In these cases, colony morphology by stereomicroscope again revealed differences (data not shown).

During the interlaboratory study rounds, seven to eight voluntary laboratories confirmed the results by using two pathways (see Figure S1 in supplementary material). Laboratories used real-time PCR for the detection of pathogenic *Y. enterocolitica* as described in ISO/TS 18867 (Anonymous 2015), Annex B, Methods 1 or 2 together with pyrazinamidase testing as described in ISO 10273, for parallel comparison to biochemical confirmation pathway of ISO 10273 (Anonymous 2017). Altogether 410 parallel confirmation reactions for 319 samples representing all study rounds gave concordant results (Table S3), thus validating the use of real-time PCR ISO/TS 18867 together with pyrazinamidase testing, in confirmation of pathogenic *Y. enterocolitica* according to ISO 10273. The option of using two alternative pathways in confirmation (see Figure S1) was therefore included in the standard revision (Anonymous 2017).

In the context of standard ISO 10273, pyrazinamidase is an important mandatory test related to virulence of *Y. enterocolitica* (Farmer et al., 1992). It was noted, however, that this test is prone to errors related to the amount of inoculum and the preparation and shelf life of ammonium iron (II) sulphate solution used in the test. Notably, the possibility of obtaining false negative results increases if limited amount of inoculum is used. Conversely, the possibility of obtaining false positive reactions increases if old ammonium iron (II) sulfate solution is used. Therefore, the amount of inoculum (generous loopful of bacteria) and the shelf-life of ammonium iron (II) sulfate solution (prepared on the day of use) is specified in the standard. Other mandatory tests related to virulence of pathogenic *Y. enterocolitica* in the revised standard are the hydrolysis of esculin and the detection of virulence plasmid (pYV) by CR-MOX agar test (Farmer et al., 1992). Complete biotyping of *Y. enterocolitica* by testing additionally for tween esterase (or lipase), fermentation of xylose and trehalose, and indole formation was left optional in the revised standard. Serotyping by the use of appropriate antisera is optional and emphasized for epidemiological purposes. However, for example for harmonised monitoring surveys for *Y. enterocolitica* in pigs, EFSA suggests that all *Y. enterocolitica* isolates are biotyped and serotyped (EFSA, 2009).

3.3.7 Cold enrichment procedure

Comparison of the performance of PSB and CEB broths by one laboratory (vegetable samples) together with the organizing laboratory (raw milk and minced meat samples) allowed the choice of using either PSB

or CEB in the cold enrichment procedure of EN ISO 10273, Annex D (data not shown). During the ILS study with lettuce samples, two laboratories tested the cold enrichment procedure and reported 15 samples positive out of 16, and 11 samples positive out of 16, respectively, by using PSB enrichment at 4°C. During the same study round, one of these laboratories also used CEB enrichment at 4°C and reported 16 samples positive out of 16. Both laboratories reported 14 samples positive out of 16 with the ILS study protocol (direct culture, PSB and ITC, see 2.2). Therefore, the cold enrichment procedure performed equal to the ILS study protocol, although this observation was based on limited data obtained during the study. As the incubation time in cold enrichment is extremely long, up to 23 days, this procedure was considered inconvenient for general use. However, it was retained in the informative Annex D of EN ISO 10273 to be used, for example, during outbreak investigations when additional procedures may be needed.

3.4 Quality assurance of the culture media

The requirement for performance testing for the quality assurance of the culture media used in the microbiological methods is universal in standardization. The productivity of ITC, PSB, CIN and nutrient agar media, and the selectivity of ITC shall be tested with the control strains and scheme specified in revised standard EN ISO 10273 Annex B (Anonymous 2017). Before the revision, the specifications were only described in ISO 11133 (Anonymous 2014) and were to be transferred in EN ISO 10273. However, during testing of the criteria in the organizing laboratory, the need for changing testing scheme and control strains was noted. The scheme was reorganised according to standard ISO 11133 Annex J instructions. The revised scheme was tested and verified by two participating independent ILS laboratories; for ITC and PSB each, two producers and four lots of media were tested, and for CIN, four producers and seven lots were tested (data not shown). As the scheme passed the testing, it was included in the standard revision (Anonymous 2017).

4 Conclusions

Performance parameters for international standard EN ISO 10273 were established in an interlaboratory study carried out by 14 laboratories in response to Mandate M/381 by European Commission. The level of detection, LOD_{50}, of the method was 9.4 CFU/25 ml in raw milk, 9.9 CFU/25 g in minced meat and 63 CFU/25 g in lettuce samples. The method showed sensitivity of 96%, 97%, and 98% in raw milk, minced meat, and lettuce samples, respectively, at inoculation levels of pathogenic *Y. enterocolitica* 5–10 times above the level of detection. The specificity was 100% in raw milk, 96% in minced meat, and 98% in lettuce.

Confirmation of colonies by real-time PCR method ISO/TS 18867 (Anonymous 2015) together with pyrazinamidase testing produced 100% concordant results with conventional biochemical confirmation. Thus, ISO/TS 18867 and pyrazinamidase testing can be used as an alternative confirmation procedure to biochemical confirmation. As every parallel step leading to the isolation of pathogenic *Y. enterocolitica* was confirmed during the trial, it was possible to evaluate the performance and necessity of parallel steps of the method. PSB enrichment and plating on CIN by using alkaline (KOH) treatment showed the highest sensitivity (52–92%, both levels of inoculation) in raw milk and minced meat samples. This was not evident in lettuce samples, in which ITC enrichment followed by KOH treatment before plating on CIN showed higher sensitivity (64% at low and 82% at high inoculation level) than plating on CIN from PSB with KOH treatment (44% at low and 74% at high inoculation level). The necessity of both enrichment broths, PSB and ITC, was supported by the statistical analysis. However, this analysis indicated no difference in the performance if steps without KOH were excluded from the ILS study protocol. Based on the analysis, plating on the CIN agar without KOH treatment could be left as optional procedure in the method.

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Tables:

Table 1. Participant laboratories' positive sample results (the maximum number of results is eight per inoculation level).

Sample matrix	Inoculation level [a]	Laboratory													
		LAB 23	LAB 24	LAB 25	LAB 26	LAB 27	LAB 28	LAB 29	LAB 31	LAB 32	LAB 34	LAB 35	LAB 36	LAB 37	LAB 39
Raw milk	Blank	0	0	0	ND	0	0	0	0	0	0	0	0	ND	0
	Low	5	7	3	ND	8	8	5	5	6	7	4	3	ND	4
	High	8	8	5	ND	8	8	8	8	8	8	6	6	ND	8
Minced meat	Blank	0	ND	2 [b]	0	0	1 [b]	0	0	0	0	0	0	0	1 [b]
	Low	5	ND	3	4	7	8	8	7	8	7	4	6	7	6
	High	6	ND	8	7	8	8	8	8	8	8	8	8	8	8
Lettuce	Blank	1 [b]	ND	0	0	1 [b]	0	0	0	0	0	0	0	0	0
	Low	8	ND	7	5	3	7	6	6	8	8	6	7	6	6
	High	8	ND	7	7	8	8	8	8	8	8	8	7	8	8

[a] Inoculation of pathogenic *Y. enterocolitica*: Blank, negative; Low, 9 CFU / 25 ml (raw milk) or 16 CFU / 25 g (minced meat) or 110 CFU / 25 g (lettuce); High, 59 CFU / 25 ml (raw milk) or 85 CFU / 25 g (minced meat) or 1 100 CFU / 25 g (lettuce). ND, no data

[b] False positive result

Table 2. Results of data analysis obtained with raw milk

Performance characteristic	**Blank** 0 CFU/25 g	**Low level contamination** 9 CFU/25 g	**High level contamination** 59 CFU/25 g
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	12 [a]	12 [a]	12 [a]
Number of samples	112	112	112
Number of samples retained after evaluation of the data	96	96	96
Sensitivity, %	–	68	96
Specificity, %	100	–	–
LOD_{50}, (95 % confidence interval) in CFU/sample	–	9.4 (7.4 to 12.0)	

[a] Two laboratories were excluded; one due to deviation in the protocol and other due to the inconsistency in the result reporting

Table 3. Results of data analysis obtained with minced meat

Performance characteristic	**Blank** 0 CFU/25 g	**Low level contamination** 16 CFU/25 g	**High level contamination** 85 CFU/25 g
Number of participating collaborators	13	13	13
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	104	104	104
Number of samples retained after evaluation of the data	104	104	104
Sensitivity, %	–	77	97
Specificity, %	96 [a]	–	–
LOD_{50}, (95 % confidence interval) in CFU/sample		9.9 (7.8 to 12.5)	

[a] False positives were obtained in three different laboratories (one laboratory 2 samples out of 8; two laboratories 1 sample out of 8)

Table 4. Results of data analysis obtained with lettuce

Performance characteristic	**Blank**	**Low level contamination**	**High level contamination**
	0 CFU/25 g	110 CFU/25 g	1 100 CFU/25 g
Number of participating collaborators	13	13	13
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	104	104	104
Number of samples retained after evaluation of the data	103 [a]	103 [a]	104
Sensitivity, %	–	81	98
Specificity, %	98 [b]	–	–
LOD_{50}, (95 % confidence interval) in CFU/sample	–	63 (49 to 81)	

[a] One sample at blank level and one sample at low level for one laboratory were excluded from analysis due to lack of confirmation tests.

[b] False positives were obtained in two different laboratories (1 sample out of 8).

Table 5. Total number of participants' positive results and samples analysed at each enrichment and plating step of the method

Enrichment/plating step	Positive results / analysed samples [a] on inoculation level [b]					
	Raw milk		Minced meat		Lettuce	
	Low	High	Low	High	Low	High
PSB 0 h/CIN (direct plating)	4 / 88 (5 %)	7 / 88 (8 %)	2 /104 (2 %)	20 / 104 (19 %)	22 / 104 (21 %)	67 / 104 (64 %)
PSB 44 h/CIN with KOH	46 / 88 (52 %)	64 / 88 (73 %)	79 / 104 (76 %)	94 / 104 (90 %)	44 / 100 (44 %)	74 / 100 (74 %)
PSB 44 h/CIN w/o KOH	15 / 88 (17 %)	21 / 88 (24 %)	3 /104 (3 %)	11 / 104 (11 %)	6 / 104 (6 %)	3 / 104 (3 %)
ITC 44 h/CIN with KOH	22 / 96 (23 %)	68 / 96 (71 %)	16 / 104 (15 %)	28 / 104 (27 %)	63 / 99 (64 %)	82 / 100 (82 %)
ITC 44 h/CIN w/o KOH	12 / 88 (14 %)	53 / 88 (60 %)	7 / 104 (7 %)	17 / 104 (16 %)	26 / 104 (25 %)	51 / 104 (49 %)
Combined results:						
PSB 44 h/CIN with KOH and w/o KOH	55 / 88 (63 %)	70 / 88 (80 %)	79 / 104 (76 %)	95 / 104 (91 %)	44 /100 (44 %)	71 / 100 (71 %)
ITC 44 h/CIN with KOH and w/o KOH	22 / 96 (23 %)	71 / 96 (74 %)	17 / 104 (16 %)	28 / 104 (27 %)	62 / 103 (60 %)	88 / 104 (85 %)
PSB and ITC 44 h/CIN with KOH	45 / 88 (51 %)	88 / 96 (82 %)	80 / 104 (77 %)	95 / 104 (91 %)	76 / 99 (77 %)	95 / 100 (95 %)
PSB and ITC 44 h/CIN w/o KOH	24 / 88 (27 %)	58 / 88 (66 %)	7 / 104 (7 %)	21 / 104 (20 %)	27 / 104 (26 %)	52 / 104 (50 %)
PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH [c]	52 / 96 (54 %)	88 / 96 (92 %)	80 / 104 (77 %)	100 / 104 (96 %)	83 / 103 (81 %)	102 / 104 (98 %)
PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH and w/o KOH [d]	65 / 96 (68 %)	89 / 96 (93 %)	80 / 104 (77 %)	101 / 104 (97 %)	83 / 103 (81 %)	102 / 104 (98 %)

[a] Samples from steps that were not confirmed were removed from study round with raw milk and lettuce

[b] Inoculation of pathogenic *Y. enterocolitica*: Blank, negative; Low, 9 CFU / 25 ml (raw milk) or 16 CFU / 25 g (minced meat) or 110 CFU / 25 g (lettuce); High, 59 CFU / 25 ml (raw milk) or 85 CFU / 25 g (minced meat) or 1 100 CFU / 25 g (lettuce).

[c] Steps represent the mandatory enrichment/plating steps of revised EN ISO 10273 (Anonymous 2017);

[d] Steps include the mandatory enrichment/plating steps of the revised EN ISO 10273:2017 (Anonymous 20017) and optional plating from enrichments PSB and ITC without KOH treatment (mandatory steps in the former version of the method, EN ISO 10273:2003, Anonymous 2003). w/o KOH: without KOH.

Table 6. Odds ratios of the treatments on the sensitivity of detection

		Posterior odds ratio [a] (95% credibility interval)			
Variable	Treatment	All ILS rounds	Raw milk	Minced meat	Lettuce
T1	PSB 0 h/CIN (direct plating)	0.1 (0.1–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.5 (0.3–0.7)
T2	PSB 44 h/CIN with KOH	-	-	-	-
T3	PSB 44 h/CIN w/o KOH	0.0 (0.0–0.0)	0.1 (0.1–0.2)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
T4	ITC 44 h/CIN with KOH	0.3 (0.3–0.4)	0.4 (0.3–0.6)	0.0 (0.0–0.0)	2.1 (1.4–3.0)
T5	ITC 44 h/CIN w/o KOH	0.1 (0.1–0.2)	0.3 (0.2–0.4)	0.0 (0.0–0.0)	0.4 (0.2–0.5)
T6	PSB 44 h/CIN with KOH and w/o KOH	1.1 (1.1–1.1)	1.2 (1.1–1.3)	1.0 (1.0–1.0)	1.0 (1.0–1.1)
T7	ITC 44 h/CIN with KOH and w/o KOH	0.5 (0.4–0.7)	0.8 (0.6–1.2)	0.0 (0.0–0.1)	3.1 (2.1–4.4)
T8	PSB and ITC 44 h/CIN with KOH	1.8 (1.6–2.0)	1.9 (1.6–2.2)	1.1 (1.1–1.2)	4.8 (3.7–6.3)
T9	PSB and ITC 44 h/CIN w/o KOH	0.2 (0.1–0.2)	0.4 (0.3–0.6)	0.0 (0.0–0.0)	0.4 (0.3–0.5)
T10	PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH	2.1 (1.9–2.4)	1.9 (1.6–2.3)	1.2 (1.1–1.3)	7.2 (5.2–9.8)
T11	PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH and w/o KOH	2.8 (2.3–3.3)	3.1 (2.4–3.9)	1.3 (1.1–1.4)	9.8 (6.8–14.0)

[a] Mean of 10 000 iterations. Odds ratios were calculated for each treatment in relation to the reference *T2*

Table 7. The effect of individual enrichment and/or plating steps and their combinations to the level of detection (LOD_{50}) within method ISO 10273

Enrichment and/or plating step	LOD_{50} (95% confidence interval), CFU/25 ml or g		
	Raw milk	Minced meat	Lettuce
Individual steps:			
PSB 0 h/CIN (direct plating)	360 (200 - 670)	300 (198 - 463)	620 (500 - 780)
PSB 44 h/CIN with KOH	21 (17 - 26)	15 (12 - 18)	380 (300 - 480)
PSB 44 h/CIN w/o KOH	100 (72 - 142)	500 (290 - 850)	9 500 (4 800 - 19 000)
ITC 44 h/CIN with KOH	31 (25 - 38)	140 (105 - 195)	250 (200 - 310)
ITC 44 h/CIN w/o KOH	44 (34 - 57)	280 (186 - 423)	820 (650 - 1 100)
Combination of steps:			
PSB 0 h/CIN and PSB 44 h/CIN with KOH	17 (14 - 21)	11 (9,1 - 14)	200 (160 - 250)
PSB 44 h/CIN with KOH and w/o KOH	15 (13 -20)	14 (11 -17)	410 (330 - 510)
ITC 44 h/CIN with KOH and w/o KOH	29 (23 - 36)	140 (103 - 190)	240 (190 - 300)
PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH [a]	12 (9,6 - 15)	11 (8,4 - 13)	68 (54 -89)
PSB 0 h/CIN, PSB and ITC 44 h/CIN with KOH and w/o KOH [b]	9.4 (7,4 - 12)	9.9 (7,8 - 13)	63 (49 to 81)

[a] Steps represent the mandatory enrichment/plating steps of the revised EN ISO 10273 (Anonymous 20017);

[b] Steps include the mandatory enrichment/plating steps of the revised EN ISO 10273:2017 (Anonymous 20017) optional plating from enrichments PSB and ITC without KOH treatment (mandatory steps in the former version of the method, EN ISO 10273:2003, Anonymous 2003)

Supplementary tables

Table S1: Homogeneity and stability of interlaboratory study (ILS) samples

				Positive samples, observed / expected [a]		
ILS round	Level	Inoculum, CFU/25 g/ml (Standard deviation, CFU)	Homogeneity of inoculum, T2 / (I - 1) [b]	Homogeneity (n = 20)	Stability, optimal [c] (n=10)	Stability, suboptimal [c] (n=10)
	Blank	0		0 / 0	0 / 0	0 / 0
	Low	9 (3,0)	0,86	17 / 1 - 20	7 / 1 - 10	6 / 1 - 10
Raw milk	High	59 (7,7)	1,33	20 / 20	10 / 10	10 / 10
	Blank	0		0 / 0	0 / 0	0 / 0
	Low	16 (4,0)	1,31	20 / 1 - 20	10 / 1 - 10	10 / 1 - 10
Minced meat	High	85 (15)	1,68	20 / 20	10 / 10	10 / 10
	Blank	0		0 / 0	0 / 0	0 / 0
	Low	110 (8,6)	0,52	20 / 1 - 20	7 [d] / 1 - 10	9 / 1 - 10
Lettuce	High	1100 (11)	1,60	20 / 20	9 [d] / 10	10 / 10

[a] Target inoculation level was at LOD_{50}, which would result approximately 5 out of 10 samples or 10 out of 20 samples positive.

[b] Acceptable variation between units of a batch: T2 / (I - 1) ≤ 2 (ISO/TS 22117:2010, Annex B)

[c] Optimal shipping conditions: Samples in transport package (containing dry ice) were kept at room temperature (22 to 25 °C) for 20 to 24 h before start of the analysis. Suboptimal shipping and storage conditions: Samples in transport package (containing dry ice) were kept at room temperature for 3 d, the package was opened and the samples transferred for storage at -20 °C for 3 or 4 d before start of the analysis.

[d)] Analysis of the sample set suffered from malfunction of KOH reagent

Table S2. Sensitivities on chromogenic media in relation to their parallel plating step on CIN agar

Enrichment/plating step	Correct positives / analysed samples [a] on inoculation level			
	Minced meat		Lettuce	
	Low	High	Low	High
PSB 44 h/CIN with KOH	79 / 104 (76 %)	94 / 104 (90 %)	44 / 100 (44 %)	74 / 100 (74 %)
PSB 44 h/CHROM [b] with KOH	NA	NA	23 / 52 (44 %)	46 / 52 (88 %)
PSB 44 h/YECA [c] with KOH	NA	NA	0 / 4 (0 %)	1 / 4 (25 %)
PSB 44 h/CIN w/o KOH	3 /104 (3 %)	11 / 104 (11 %)	6 / 104 (6 %)	3 / 104 (3 %)
PSB 44 h/CHROM w/o KOH	NA	NA	1 /32 (3 %)	1 /32 (3 %)
PSB 44 h/YECA w/o KOH	NA	NA	NA	NA
ITC 44 h/CIN with KOH	16 / 104 (15 %)	28 / 104 (27 %)	63 / 99 (64 %)	82 / 100 (82 %)
ITC 44 h/CHROM with KOH	11 / 56 (20 %)	12 / 56 (21 %)	2 / 8 (25 %)	5 / 8 (63 %)
ITC 44 h/YECA with KOH	3 / 51 (6 %)	7 / 48 (15 %)	NA	NA
ITC 44 h/CIN w/o KOH	7 / 104 (7 %)	17 / 104 (16 %)	26 / 104 (25 %)	51 / 104 (49 %)
ITC 44 h/CHROM w/o KOH	4 / 56 (7 %)	3 / 56 (5 %)	0 / 8 (0 %)	0 / 8 (0 %)
ITC 44 h/YECA w/o KOH	3 / 71 (4 %)	11 / 68 (16 %)	NA	NA

[a] Samples from steps that were not confirmed were removed from analysis. NA: Not analysed.

[b] CHROM: CHROMagar™ *Y. enterocolitica* (CHROMagar, Paris, France) (Renaud et al. 2013);

[c] YECA agar (bioMérieux, Marcy l'Etoile, France) (Denis et al. 2011)

Table S3. Comparison of colony confirmation by real-time PCR method [a] and biochemical confirmation pathway [b]

Performance characteristic	No. of laboratories in the study	No. of samples with parallel confirmation [c]	No. of parallel confirmation reactions	No. of inconsistent results
Confirmation results / raw milk	7	91	119	0
Confirmation results / minced meat	8	122	130	0
Confirmation results / lettuce	8	106	161	0
Confirmation results / all matrices		319	410	0

[a] ISO/TS 18867:2015 (Anonymous 2015), Annex B, Methods 1 or 2 and pyrazinamidase testing

[b] Revised ISO 10273 (Anonymous 2017)

[c] See Figure S1 for the further explanation of alternative confirmation pathways.

Figure S1

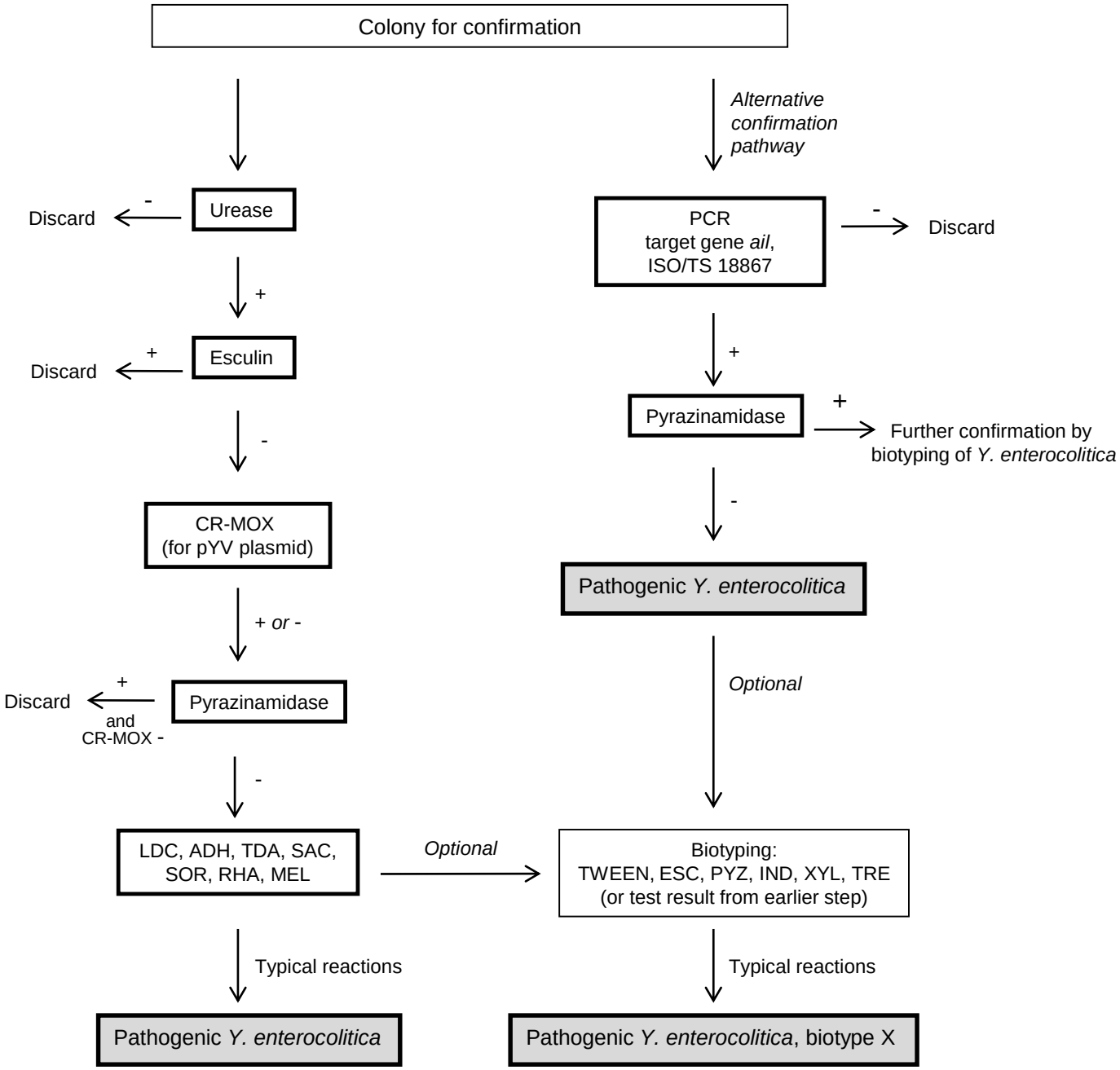
Colony for confirmation
Alternative confirmation pathway
Urease
-
Discard
+
Esculin
+
Discard
-
CR-MOX (for pYV plasmid)
+ or -
Pyrazinamidase
+
Discard
and CR-MOX -
-
LDC, ADH, TDA, SAC, SOR, RHA, MEL
Optional
Typical reactions
Pathogenic Y. enterocolitica
PCR target gene ail, ISO/TS 18867
-
Discard
+
Pyrazinamidase
+
Further confirmation by biotyping of Y. enterocolitica
-
Pathogenic Y. enterocolitica
Optional
Biotyping: TWEEN, ESC, PYZ, IND, XYL, TRE (or test result from earlier step)
Typical reactions
Pathogenic Y. enterocolitica, biotype X

Figure S1 caption

Figure S1. Diagram of the confirmation of pathogenic *Y. enterocolitica*

Figure S2

Click here to download high resolution image

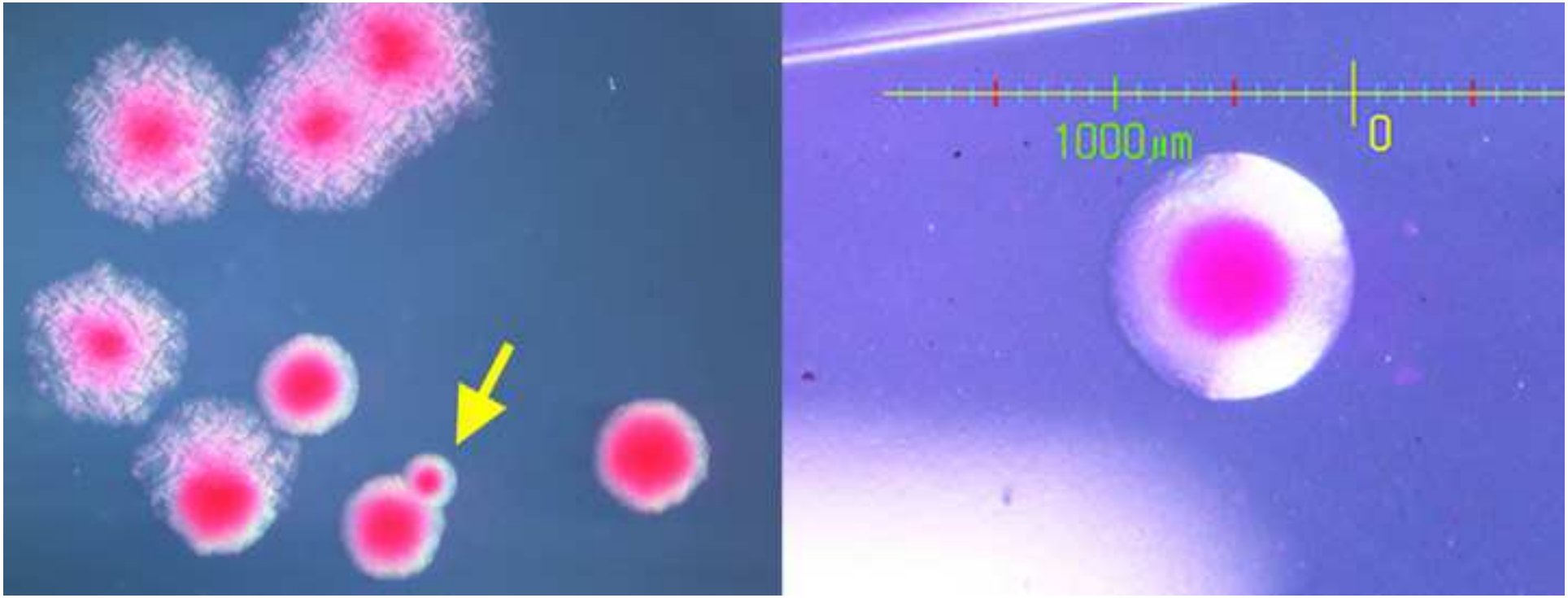

Figure S2 caption

Figure S2. Use of stereomicroscope in identification of pathogenic *Y. enterocolitica* colonies on CIN agar (incubation at 30°C for 24 h). Left panel: one suspected colony of pathogenic *Y. enterocolitica* (indicated by arrow) among colonies of background microbiota (incl. non-pathogenic *Yersinia*) through stereomicroscope. On CIN agar, pathogenic *Y. enterocolitica* appear as small (approximately 1 mm) colonies with entire, circular edge. Right panel: the characteristic morphology of the *Y. enterocolitica* bioserotype 4/O:3 colony can be identified in the magnification of the pure culture of the suspected colony on CIN. The colonies have a small, deep red sharp bordered centre (“bull’s eye”). The surrounding rim is translucent or transparent and, when examined with obliquely transmitted light, non-iridescent and finely granular.