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A pan-European ring trial to validate an International Standard for detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafoods

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ABSTRACT

Globally, vibrios represent an important and well-established group of bacterial foodborne pathogens. The European Commission (EC) mandated the Comite de European Normalisation (CEN) to undertake work to provide validation data for 15 methods in microbiology to support EC legislation. As part of this mandated work programme, merging of ISO/TS 21872–1:2007, which specifies a horizontal method for the detection of *V. parahaemolyticus* and *V. cholerae*, and ISO/TS 21872–2:2007, a similar horizontal method for the detection of potentially pathogenic vibrios other than *V. cholerae* and *V. parahaemolyticus* was proposed. Both parts of ISO/TS 21872 utilized classical culture-based isolation techniques coupled with biochemical confirmation steps. The work also considered simplification of the biochemical confirmation steps. In addition, because of advances in molecular based methods for identification of human pathogenic *Vibrio* spp. classical and real-time PCR options were also included within the scope of the validation. These considerations formed the basis of a multi-laboratory validation study with the aim of improving the precision of this ISO technical specification and providing a single ISO standard method to enable detection of these important foodborne *Vibrio* spp.. To achieve this aim, an international validation study involving 13 laboratories from 9 countries in Europe was conducted in 2013. The results of this validation have enabled integration of the two existing technical specifications targeting the detection of the major foodborne *Vibrio* spp., simplification of the suite of recommended biochemical identification tests and the introduction of molecular procedures that provide both species level identification and discrimination of putatively pathogenic strains of *V. parahaemolyticus* by the determination of the presence of theromostable direct and direct related haemolysins. The method performance characteristics generated in this have been included in revised international standard, ISO 21872:2017, published in July 2017.

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1. Introduction

Vibrios are Gram-negative rod-shaped bacteria that are natural constituents of estuarine and marine environments (Baker-Austin et al., 2016). The genus *Vibrio* contains over 100 described species, and around a dozen of these have been demonstrated to cause infections in humans (Austin, 2005). Typically, *Vibrio* infections are initiated from exposure to seawater or consumption of raw or undercooked seafood produce (Altekruse et al., 2000; Iwamoto et al., 2010). The species most commonly associated with foodborne infections include *V. parahaemolyticus, V. vulnificus* and *V. cholerae*. Several factors underline the importance of these foodborne pathogens from an international perspective. Around 50–85,000 foodborne *Vibrio* infections are believed to occur each year in the USA, based on extrapolation data from active and passive epidemiological surveillance systems (Scallan et al., 2011). Compared to other major foodborne pathogens, the number of *Vibrio* infections also appears to be steadily increasing (Martinez-Urtaza et al., 2010; Newton et al., 2012). Recent data from the Centers for Disease Control and Prevention (CDC) indicate that foodborne infections associated with these bacteria have increased significantly in the USA (Newton et al., 2012). Notably, of all the major bacterial foodborne pathogens (e.g. *Salmonella*, *Listeria*, *Escherichia coli* O157 and *Camplyobacter*), vibrios are the only group that are currently increasing in incidence in the USA. Seafood-associated outbreaks are now being reported in geographical areas where these bacteria were once considered absent (Baker-Austin et al., 2016; Martinez-Urtaza et al., 2010; Gonzalez-Escalona et al., 2005; Martinez-Urtaza et al., 2013). There is a growing body of evidence to suggest that climate change, coupled to epidemiological and demographic factors are increasing the geographical spread as well as incidence of these pathogens from foodborne sources (Baker-Austin et al., 2016; Martinez-Urtaza et al., 2010; Vezzulli et al., 2013).

Currently there are no European Union (EU) regulatory microbiological criteria for *Vibrio* spp. in seafood products traded between Member States or for third country imports. In 2010 Codex published guidelines on the application of general principles for vibrios and seafood produce (CAC/GL 73–2010). However, Codex did not provide definitive microbiological criteria, but specified the need for improvements for microbiological approaches in this area. The EU Regulation (EC) No. 2073/2005 sets out the microbiological criteria for foodstuffs produced and traded in Europe, but there are no specific microbiological criteria for *Vibrio* spp. in this regulation. Although some unilateral risk based controls for imported seafood have been adopted, in principle these are carried out in an *ad hoc* manner (Baker-Austin et al., 2010). In part the lack of introduction of EU standards for *Vibrio* spp. in seafoods has been due to the lack of suitable, validated discriminatory methods for *Vibrio* spp. of major foodborne significance (e.g. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Scallan et al., 2011)), and specifically for toxigenic strains of *V. parahaemolyticus*. The European Commission mandated the Comite de European Normalisation (CEN) to undertake work to provide validation data for 15 methods in microbiology to support EC legislation. Amongst these were the 2 existing EN ISO Technical Specifications for the detection of *Vibrio* spp. of potential public health significance – ISO/TS 21872 parts 1 and 2. ISO/TS 21872–1 provided a detection method for *V. cholerae* and *V. parahaemolyticus*, whereas ISO/TS 21872–2 entitled 'Detection of potentially pathogenic *Vibrio* spp. other than *V. cholerae* and *V. parahaemolyticus* essentially set out a method for detection of *V. vulnificus*. Both ISO/TS 21872 parts 1 and 2 were classical culture based qualitative microbiological methods with a suite of biochemical tests for isolate confirmation.

Following preliminary practical evaluation at the European Union Reference Laboratory (EURL), a consultation with AFNOR sub group on *Vibrio* methods and discussion with experts amongst EU National Reference Laboratory (NRLs) was initiated. A number of improvements to ISO/TS 21872 parts 1 and 2 were proposed to the relevant sub committees at International Standards Organisation (ISO) and CEN (ISO SC9 and CEN WG6) and subsequently agreed by DG Sante and DG Entr. These comprised merging the two parts to a single standard focusing on major foodborne species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, a revision to include provision for use of PCR based identification of the three species, thermostable direct haemolysin (*tdh*) and direct related haemolysin (*trh*) genes that allow the differentiation of pathogenic strains of *V. parahaemolyticus* and minor amendments to biochemical confirmatory tests. A revised standard ISO 21872 was accepted as a New Work Item Proposal (NWIP) by CEN WG6 and this was within the scope of the validation. This study aimed to validate the consolidated aspects of ISO 21872, utilising collaborative trials of two different seafood matrices (raw bivalve molluscan shellfish and frozen prawns) in order to replace the technical specification with a full, validated EN/ISO standard.

2. Materials and methods

2.1. Selection of target regions for PCR based species and strain confirmation

A preliminary collaborative trial amongst 23 NRLs for monitoring viral and bacteriological contamination of bivalve molluscs and other specialist laboratories across the EU was conducted to assist with selection of molecular targets for *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. This included selection and verification of species markers and molecular targets to enable the detection of putative pathogenic strains of *V. parahaemolyticus* possessing *tdh* and *trh* genes. Samples were distributed as swabs; reference strains of target and non-target *Vibrio* spp. were included in the study. A method protocol including well established primer sets and specific running conditions were produced to accompany test samples. Test samples were anonymised and distributed as semi-solid marine agar swabs inoculated with reference strains of target and non-target *Vibrio* spp.. Swabs were inoculated into 225 ± 5 mL Alkaline Salts Peptone Water (ASPW) and subject to primary enrichment at 41.5 ± 1 °C and 37 ± 1 °C for 6 ± 1 h. One millilitre aliquots of each enrichment were subject to secondary enrichment in 10 ± 0.5 mL fresh ASPW at 41.5 ± 1 °C and 37 ± 1 °C for 18 ± 3 h. Following primary and secondary enrichment, 1 μL of each enrichment broth were streaked onto the surface of Tris Citrate Bile Salts (TCBS) and a second plating medium. TCBS plates were incubated at 37 ± 1 °C for 24 ± 3 h; second plating media were incubated according to the manufacturers' instructions. A minimum of two colonies showing typical phenotypic characteristics of *Vibrio* spp. were sub-cultured onto Saline Nutrient Agar and incubated at 37 ± 1 °C for 24 ± 3 h. Subsequent cultures were checked visually for purity and subject to oxidase tests and microscopic examination (motility and Gram stain). Oxidase positive, Gram negative, motile isolates were subject to tests for glucose utilisation, lactose and sucrose fermentation, lysine decarboxylation, β-galactosidase activity, presence of arginine dihydrolase, indole production, ornithine decarboxylase production and growth in 0, 2, 6, 8, and 10% NaCl. In parallel, DNA was extracted from a single colony suspended in 500 μL of nuclease free water, the bacterial suspension was heated at 95 ± 1 °C for 5 ± 1 mins and centrifuged at 10,000 × *g*. The resultant supernatant was stored at < −15 °C for PCR analysis. Aliquots of 2.5 μL of extracted DNA were added to a mastermix containing 10 μL reaction buffer, 5 μL $MgCl_2$, 0.625 μL dNTPs (20 mM), 0.5 μL primer (forward and reverse), 0.25 μL Taq polymerase and 30.625 μL nuclease free water. All samples were subjected to PCR according to the cycling parameters described in for *ToxR* (Kim et al., 1999), *tdh* and *trh* (Bej et al., 1999), *prVC* (Chun et al., 1999) and *VVH* (Hill et al., 1991). Products were visualised on 2% agarose gels following electrophoresis at 130 V for 25–30 min.

2.2. Selection of laboratories for ring trials

Recruitment of collaborating laboratories was by open competition.

An open call for participants' was launched via the Cefas and EURL websites giving a description of the scope of the proposed work and inviting expressions of interest. Laboratory selection was made by an EURL expert panel and was based upon demonstrable competencies according to the following criteria: 1) demonstrable experience in working with *Vibrio* spp. and bacteriological testing of seafoods; 2) participation in formal external quality assurance schemes for *Vibrio* spp. and 3) familiarity with quality assurance procedures and method standardisation.

3. Collaborative trial 1 - matrix raw bivalve molluscan shellfish

3.1. Generation of samples, distribution and quality control

Eight hundred Pacific oysters (*Crassostrea gigas*) were obtained from a commercial fishery in the UK, and the animals were depurated (purified by holding in 'clean' seawater for 48 h) at the organizing laboratory. Following depuration oysters were removed from tanks and shucked (opened) aseptically. The flesh and intravalvular fluid was homogenised, pooled and split into three large sterile mixer bowls to produce master homogenates. Samples were then spiked with log phase bacterial cultures as follows: 1) *V. vulnificus* WDCM 00139 low level 2×10 CFU/25 g, 2) *V. parahaemoltycus* EURL V05/14 high level 2×10^6 CFU/25 g, and 3), *V. parahaemoltycus* EURL V05/14 low level 2×10 CFU/25 g. Master homogenates were mixed thoroughly using a food processor and 35 mL volumes were aliquoted into 50 mL Falcon tubes. Samples were packed in 10 L biotherm units with cool packs and distributed by courier under refrigeration conditions according to UN 3373 packing instructions. Samples comprised 8 replicate samples uncontaminated homogenates, 8 samples with inoculated low *V. vulnificus* and high *V. parahaemolyticus*, and 8 samples inoculated with low levels of *V. parahaemolyticus*. The distribution took place on 18th July 2013, thirteen laboratories were sent blind samples labelled 1–24. All samples arrived at their destination within 48 h of dispatch. Separately all participating laboratories received primers, probes and positive and negative control material, as bacterial DNA on dry ice.

4. Collaborative trial 2 - matrix cooked, frozen prawns

4.1. Generation of samples, distribution and quality control

Five kg of cooked, frozen prawns (*Penaeus* spp.) were purchased from a commercial retail establishment. Samples were defrosted, homogenised (1:2 with 0.1% peptone) and split into three large sterile mixer bowls to produce master homogenates. Master homogenates were then split into 35 mL volumes in sterile 50 mL Falcon tubes and frozen at < -15 °C. Each laboratory received 24 samples packed in 15 L biotherm units with freezer packs and distributed by courier according to UN 3373 packing instructions. The distribution took place on 18th November 2013, to twelve laboratories. Freeze dried cultures were dispatched separately to laboratories with instructions on reconstitution of freeze dried cultures and inoculation of samples prior to analysis. All samples arrived at their destination within 48 h of dispatch. Separately all participating laboratories received primers, probes and positive and negative control material, as bacterial DNA on dry ice. Target levels for inocula were: 1) *V. vulnificus* $\approx 1 \times 10^2$ CFU/25 g low level, high level 1×10^4 CFU/25 g, 2) *V. parahaemolylicus* 1×10^3 CFU/25 g (low/medium level) and 3) *V. cholerae* 1×10^3 CFU/25 g low level, high level 1×10^5 CFU/25 g (low/medium level).

5. Testing procedures used at different participating laboratories and analysis of data

Laboratories involved in the validation exercise were provided with a standard operating procedure (SOP) outlining the steps required for the isolation, identification and verification of strains obtained in distributions 1 (raw bivalve shellfish) and 2 (prawns). In brief, the SOP provided information on the preparation and first enrichment of samples, secondary enrichments, isolation and confirmation of pure cultures, biochemical confirmation and verification using conventional and real-time PCR assays. The SOP also provided a template for the expression of results. The SOP was based on draft iterations of ISO/TS 21872, with minor modifications (Fig. 1). The PCR and real-time PCR methods described in the SOP and used for confirmatory purposes were based on a range of well-established published assays for *V. cholerae* (Chun et al., 1999), *V. parahaemolyticus* (Kim et al., 1999; Bej et al., 1999) (including putative pathogenicity maker genes *tdh* and *trh*) (Bej et al., 1999; Nordstrom et al., 2007) and *V. vulnificus* (Hill et al., 1991; Campbell and Wright, 2003 (with minor modification)) thus meeting the criterion established in the draft standard that primer (and hydrolysis probe) sequences shall be published in peer-review journals and verified against a broad range of target and non-target strain running conditions. PCR set up and primer and probe sequences were subsequently based on these published studies. Data returned to the EURL for analysis were subsequently collated and analysed per distribution, with performance characteristics gathered. Sensitivity and specificity were calculated according to the following criteria. The sensitivity was defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results were thus dependent on the level of contamination of the sample. The specificity was defined as the number of blank samples found negative divided by the number of blank samples tested found negative. Criteria for determining both specificity and sensitivity were defined by ISO statistics working group and were consistent with other published ISO documents in mandate M381. Currently estimated as per g, 95% confidence intervals in parentheses. Performance characteristics such as limit of detection (LOD) and LOD_{50} of submitted data from participating laboratories was analysed using a Probability of Detection (POD) approach essentially as previously described (Wilrich and Wilrich, 2009) these were estimated per g with 95% confidence intervals. The values of the performance characteristics derived from this interlaboratory study are shown per bacterial determinant and sample type in Tables 1–3. Identification of target *Vibrio* spp. was by biochemical, conventional and/or real-time PCR. Real-time PCR for identification of *V. cholerae* was tested in this interlaboratory study but data generated were not reliable and subsequently omitted (data not shown).

6. Results

6.1. Selection of laboratories for ring trials

Twenty-three laboratories were recruited into the initial EURL trials to select molecular target regions for identification of *V. parahaemolyticus*, *tdh*, *trh* genes, *V. vulnificus* and *V. cholerae*. Of these, 15 responded to the open call for participation in the validation exercise. Thirteen laboratories were selected from assessment of the expressions of interest provided detailing their relevant experience against the selection criteria. Two laboratories were not considered to have demonstrated sufficient experience in the application of molecular methods for the detection of foodborne bacterial pathogens or did not have demonstrable experience of relevant quality assurance or standardisation. Consequently, 13 laboratories were selected for inclusion in the validation.

6.2. Collaborative trial 1 - matrix raw bivalve molluscan shellfish

A large dataset was generated from the interlaboratory trial exercises carried out as part of the CEN validation study. These data were used to determine the performance characteristics of the method in matrix samples. In the evaluation of these data, biochemical and molecular isolate identification approaches were considered together. In a small number of cases isolate identification using biochemical and

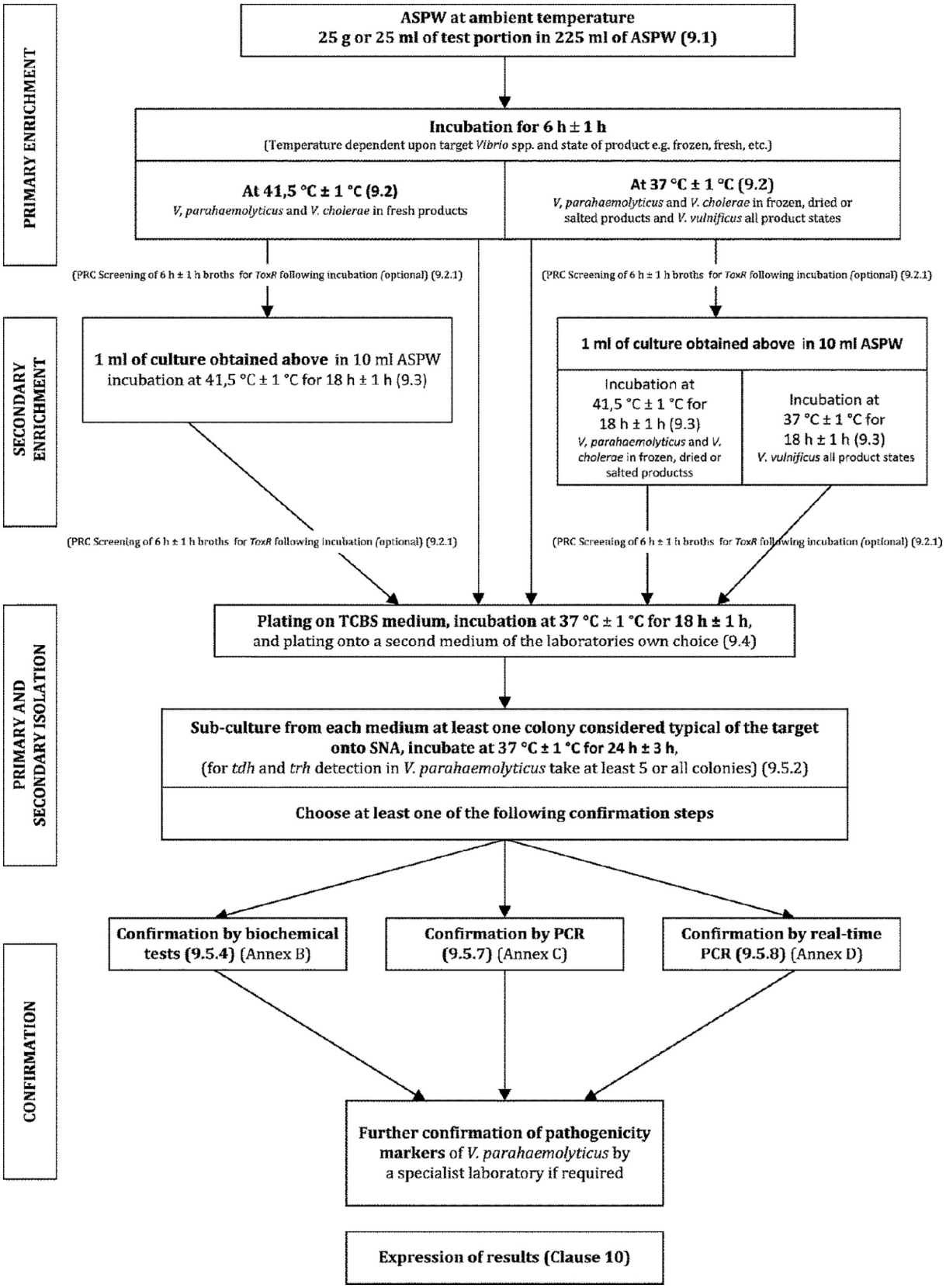

Fig. 1. Diagram of procedure for the detection of enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*.

molecular approaches did not yield corresponding results (data not shown). In these cases, samples were considered positive if the laboratory recorded that either one of the colony identification approaches employed following primary and secondary enrichment at either 37 °C or 41.5 °C yielded a positive result. This approach was also employed for assessment of Collaborative trial 2. Thirteen participating laboratories took part in the raw bivalve mollusc distribution (Tables 1–3). This number was reduced to 10 participating laboratories

Table 1
Results of data analysis obtained with *Vibrio parahaemolyticus* and *Vibrio vulnificus* in raw oysters.

Parameters	*Vibrio parahaemolyticus*			*Vibrio vulnificus*	
	Contamination level			Contamination level	
	Blank	Low (2×10 CFU/25 g)[a]	High (2×10^6 CFU/25 g)[a]	Blank	Low (2×10 CFU/25 g)[a]
Number of participating laboratories	13	13	13	13	13
Number of retained participating laboratories after evaluation of the data	10	10	10	10	10
Number of samples	104	104	104	104	104
Number of samples retained after evaluation of the data	80	80	80	104	80
Sample size (g/mL/cm2/item)	25	25	25	25	25
Sensitivity, %[b]	90	72.5	100	90	37.5
Specificity, %[b]	111			77.5	
LOD_{50}, cfu/g[c]	0.43 (0.32 to 0.57)			12.31 (8.46 to 17.91)	

[a] Inocula levels prior to inoculation were estimated using optical density measurements at 600 nm with reference to previously prepared growth curves for each strain under test, confirmation of inoculum was by a 100 µL spread plate method on marine agar in triplicate.

[b] Sensitivity and specificity were calculated according to the following criteria. The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample. The specificity is defined as the number of blank samples found negative divided by the number of blank samples tested found negative. Sensitivity and specificity were calculated and determined where specificity exceeds 100% where positive identifications were reported in 'blank' samples.

[c] LOD_{50} was calculated using the methods described elsewhere (ISO C, 2016). Currently estimated as per g, 95% confidence intervals in parentheses.

Table 2
Results of data analysis obtained for *tdh* and *trh* for *Vibrio parahaemolyticus* in raw oysters.

Parameters	Confirmation tests[a]		
	Conventional PCR		Real-time PCR
	tdh	*trh*	*tdh*
Number of participating laboratories	6	7	9
Number of retained participating laboratories after evaluation of the data	6	7	9
Sensitivity, %[c]	86	94	73
Specificity, %[b]	89	91	94

[a] PCR methods (real time or conventional PCR applied to isolated presumptive/confirmed *V. parahaemolyticus* colonies ($n \leq 5$) only from up to 16 samples per laboratory of *V. parahaemolyticus*) not all laboratories used both conventional and real-time methods for assignment of *tdh* and *trh*.

[b] Calculated as number of isolates reported positive for either *tdh* or *trh* genes divided by number of isolates tested.

[c] Sensitivity not considered adequate from data returned from participants, real-time PCR identification of *trh* genes was not considered reliable.

following the evaluation of data. Entire datasets were excluded from 3 laboratories, as complete sets of results for both biochemical and molecular identification procedures for isolated colonies were not provided. Eighty samples of low-level spiked material and high-level spiked material (*V. parahaemolyticus*) and eighty blank samples were used for data appraisal purposes (Table 1). The highest % sensitivity results obtained were in the high-level contamination sample (2×10^6 CFU/25 g *V. parahaemolyticus*) where 100% of samples gave positive results. Lower level contamination samples (2×10 CFU/25 g *V. parahaemolyticus*) generated positive results in almost three quarters of tested samples (72.5%), with blank samples indicating sensitivity levels of 90%. Specificity was calculated at 111% across all samples, exceedance of 100% for specificity resulted from a low number of positive identifications in "blank" samples (Table 1). The LOD_{50} (CFU/g) was determined to be 0.43, with a range of 0.32 to 0.57. Lower overall levels of sensitivity were observed using testing methods for *V. vulnificus*, which generated positive results in only 37.5% of samples from the low-level contamination scenario (2×10 CFU/25 g *V. parahaemolyticus*, Table 1). This observation was corroborated following calculation of the LOD_{50} which showed a notably higher obtained value (12.31 CFU/g – range 8.46 to 17.91) compared to similar inocula levels for *V.*

Table 3
Results of data analysis obtained with *Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulnificus* in cooked prawns.

Parameters	*Vibrio cholerae*			*Vibrio vulnificus*			*Vibrio parahaemolyticus*	
	Contamination level			Contamination level			Contamination level	
	Blank	Low (1×10^3 CFU/25 g)[a]	High (1×10^5 CFU/25 g)[a]	Blank	Low (1×10^2 CFU/25 g)[a]	High (1×10^4 CFU/25 g)[a]	Blank	Low (1×10^3 CFU/25 g)[a]
Number of participating collaborators	11	11	11	11	11	11	11	11
Number of collaborators retained after evaluation of the data	10	10	10	10	10	10	10	10
Number of samples	88	88	88	88	88	88	176	88
Number of samples retained after evaluation of the data	80	80	80	80	80	80	128	64
Sensitivity, %[b]	100	92.5	92.5	95	61.3	71.5	100	100
Specificity, %[b]	84			60			99	
LOD_{50}, CFU/g[c]	11.34 (6.26 to 20.56)			81.2 (63.4–101.2)				

[a] Bacterial inocula prepared as freeze-dried ampoules supplied by FEPTU, PHE, UK according to organizing laboratories specification. All *V. parahaemolyticus* tested by conventional PCR (Fig. 1) and real-time PCR (Fig. 1) for *tdh* and *trh* genes gave negative results, inoculum intended result *tdh* and *trh* negative.

[b] Sensitivity and specificity were calculated according to the following criteria. The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample. The specificity is defined as the number of blank samples found negative divided by the number of blank samples tested found negative.

[c] LOD_{50} was calculated using the methods described elsewhere (ISO C, 2016). Currently estimated as per g, 95% confidence intervals in parentheses.

parahaemolyticus. Irrespective, good species specificity was obtained, with 77.5% of samples providing expected results. An additional aspect of the validation exercise was to obtain relevant performance characteristics of tests to determine virulence markers used for *V. parahaemolyticus*, using published assays for both conventional and real-time PCR assays for *tdh* and *trh*. A smaller subset of participating laboratories provided results for detection of *tdh* and *trh* genes in strains isolated from the main validation samples (9 laboratories provided data for real-time PCR and 7 for conventional PCR), the data indicate that both methods perform well in terms of discriminating potentially pathogenic strains based upon the presence of either or both thermostable haemolysin genes (Table 2). Similar levels of % specificity and sensitivity were observed for conventional PCR methods for *tdh* and *trh* (around 90%), although slightly lower sensitivity was observed using real-time PCR for *tdh* (Table 2). The real-time *trh* assay used during this validation exercise did not perform reliably and was subsequently omitted from the final dataset.

6.3. Collaborative trial 2 - matrix cooked, frozen prawns

Eleven of the original thirteen participating laboratories undertook a second distribution for detection of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* in cooked frozen prawns. Of the eleven datasets returned, data from one laboratory was excluded from the assessment as substantive deviations from protocols for real-time identification were reported. Analysis of the remaining dataset revealed good sensitivity (92.5% in both low and high level spiked samples), and good specificity (84%) for *V. cholerae* with LOD_{50} results (11.34 CFU/25 g – range 6.26 to 20.56) (very similar to the reported LOD_{50} for *V. vulnificus* in raw oysters). No *V. cholerae* strains were reported from blank samples of prawns, giving sensitivity % of 100% (Table 3). Less satisfactory performance was obtained in prawn samples spiked with either low (1×10^2 CFU/25 g) or high (1×10^4 CFU/25 g) levels of *V. vulnificus* (Table 3) where assessment indicated sensitivity of between 61.25 and 71.5% in low and high level contaminated samples respectively and specificity of 60%. A substantively higher LOD_{50} (81.2 CFU – range 63.39–101.2) was also observed in these samples. These data contrast with the performance characteristics obtained for *V. parahaemolyticus* in cooked, frozen prawns (Table 3), which showed 100% sensitivity in blank and low-moderate level (1×10^3 CFU/25 g) spiked samples and demonstrated that *V. parahaemolyticus* could be accurately identified in 99% of tested samples (Table 3).

7. Discussion

Globally, the species *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* represent the most important bacterial pathogens associated with the consumption of seafood produce (Newton et al., 2012; Morris, 2003; Potasman et al., 2002). In many countries, a food safety criteria or guidance have been introduced to limit the risks of exposure of consumers of seafood to *Vibrio* spp. (Toyofuku, 2014; FDA, 2015; Baker-Austin et al., 2010). Some such interventions are based upon the introduction of testing regimes that determine food safety criteria for *Vibrio* spp. in certain foodstuffs. In the EU 2001, the former Scientific Committee on Veterinary Measures Related to Public Health (now European Food Safety Authority (EFSA)) published an opinion on *V. parahaemolyticus* and *V. vulnificus* in raw seafood (SCVMPH, 2001). With respect to methods, it was concluded that the sensitivity, specificity and pathogenicity determination of the different methods in use was highly variable and thus the results of studies were difficult to compare. It was recommended *inter alia* that the elaboration of methods that enabled detection, enumeration and virulence characterisation of *V. parahaemolyticus* and *V. vulnificus* in seafoods should be a priority. Such methods were required in order to enable robust assessment of public health risks associated with *Vibrio* spp. in seafoods, which may ultimately be used to inform decisions on efficacy of control measures or to support future microbiological criteria. This validation exercise therefore was intended, in part, to generate data to enable improvements to existing ISO TS for *Vibrio* spp., which would target the primary *Vibrio* spp. of human health relevance in seafoods (*V. parahaemolyticus* and *V. vulnificus*) and enable virulence characterisation of *V. parahaemolyticus*. In addition, because there is clear evidence that *V. cholerae* can be associated with consumption of seafoods (Donovan and van Netten, 1995; Barrow and Feltham, 1993), and its detection was included within ISO/TS 21872–1, it was also incorporated into the scope of the validation exercise.

The objective of this work was to combine and simplify ISO/TS 21872 Microbiology of the food and animal feeding stuffs – Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. Part 1 Detection of *V. parahaemolyticus* and *Vibrio cholerae* and Part 2 Detection of species other than *V. parahaemolyticus* and *Vibrio cholerae* and, to introduce the ability to use molecular identification approaches following colony isolation from seafood matrices. The introduction of molecular approaches was intended to enable both identification of target *Vibrio* spp. to the species level, and to facilitate detection of the putative pathogenicity markers *tdh* and *trh* of *V. parahaemolyitcus*. Many authors have reported on the difficulties in the interpretation of biochemical identification methods for *Vibrio* spp. from environmental sources (including those deriving from seafoods) (Alsina and Blanch, 1994; Janda et al., 1988; Croci et al., 2007; Brenner et al., 2005; Ottaviani et al., 2003), and it has long been recognised that methods for vibrios of human health relevance require improvements and refinements (Baker-Austin et al., 2010), particularly given their emerging nature internationally. The work presented here provides validation data to support the elaboration of a single standardised methodological framework for the detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in a range of seafood matrices.

Simplification of recommended number of biochemical identification tests included in the revision enables laboratories to maximise resources and to target the tests which provide the most discriminatory abilities. Atypical biochemical test results amongst *Vibrio* spp. isolated from the environment have been reported by several authors (Alsina and Blanch, 1994; Croci et al., 2007). For *V. parahaemolyticus* and *V. vulnificus* positive results for production of decarboxylase enzymes (ornithine decarboxylase test) can be variable, and are influenced by the effects of salt concentration in plating media (Brenner et al., 2005). For targeting of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, it was considered that inclusion of lysine decarboxylase and arginine dihydrolase tests provided sufficient discriminatory ability. Similarly, sugar fermentation patterns based on interpretation of the saline triple sugar iron agar test did not generate additional lucidity in the discrimination of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (data not shown), with substantial variability in positivity reported previously for *V. vulnificus* (Barrow and Feltham, 1993).

The results of the validation exercise verified the utility of a number of the species-specific testing reliant on PCR (or real-time PCR) that had been previously published and that had performed well in earlier EURL ring trials. Although not considered explicitly within the scope of the validation, which considered either biochemical or molecular isolate identification as permissible, the data indicated that for identification of colonies using molecular species identification methods for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* were less subjective and required less specialist experience to interpret. This finding is in accordance with numerous reports supporting the use of molecular identification for these cryptic species, especially in respect to environmental isolations (Kim et al., 1999; Bej et al., 1999; Hill et al., 1991; Campbell and Wright, 2003; Nordstrom et al., 2007; Barrow and Feltham, 1993; Croci et al., 2007; Chen et al., 2003). Whilst molecular identification methods were broadly successful, problems were identified with 2 real-time PCR assays selected for inclusion within the validation (real-time PCR assays for *V. cholerae* (Lyon, 2001) and *trh* for *V. parahaemolyticus* (Nordstrom et al., 2007)), where data demonstrated

poor performance methodologies were excluded from the annexes of the published ISO standard as the methods could not be recommended on the basis of the validation. Further work in determining the factors underlining the poor performance is required to elucidate this fully. The validation data demonstrating the utility of *trh* and *tdh* PCR testing methods (Table 2) using methods described previously (Bej et al., 1999) represents a significant enhancement to the existing TS in that it enables non-specialist/non-reference laboratories to discriminate between pathogenic and non-pathogenic strains of *V. parahaemolyticus*. Adoption of this approach will enable more rapid assessment of the potential public health risks associated with isolation of *V. parahaemolyticus* in seafoods.

The data generated in this multi-laboratory study enabled the publication of performance characteristics (sensitivity, specificity and LOD_{50}). Concordance, i.e. the percentage of all data pairings of duplicates giving the same results used as a measure of reproducibility for qualitative analysis, was calculated according to ISO 16140:2003 (Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods). For the two trials, concordance was calculated as 87.7%, 62.5% and 92.5% for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* respectively. Performance data *Vibrio* spp. is sparse, however, for *V. parahaemolyticus* and *V. cholerae* $LOD_{50,}$ sensitivity and specificity for seafoods matrices were broadly similar to other bacterial – matrix combinations reported elsewhere (FAO/WHO, 2017). For *V. vulnificus* method performance was below that generally expected for bacteriological culture based methods with respect to LOD_{50}. This finding was not surprising and confirms the assertions in a number of studies that report the difficulties in isolation of *V. vulnificus* from environmental samples containing mixed competing microbial flora (reviewed in (Harwood et al., 2004).

In conclusion, the data generated from this pan-European ring trial to validate an international standard method for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafoods represent an important technical advance. This work allows for a more streamlined and simplified standard method that allows for the first time, the simultaneous identification of three *Vibrio* spp. of major foodborne significance. This advance also improves the accuracy of identification, the simplification of biochemical tests and introduction of molecular species markers for these pathogens. Critically, for the first time, this method facilitates non-specialist and/or non-reference laboratories to discriminate between pathogenic and non-pathogenic strains of *V. parahaemolyticus*. This method does not, however provide a basis for the quantitative evaluation of samples so, in the case of *V. parahaemolyticus*, or in situations where enumeration of toxigenic strains is deemed important, further methodological work is clearly required. Current work involves refining a quantitative approach, based on real-time PCR coupled to MPN based format (Cantet et al., 2013), as a possible quantitative testing platform.

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