European Union Reference Laboratory (EURL) Proficiency Testing Scheme

Development of PCR (conventional and real-time) for the detection of *Vibrio* spp.

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Introduction

Vibrios are Gram-negative bacteria which a number are pathogens commonly encountered in warm estuarine and marine environments. Certain species of vibrios, such as *V. parahaemolyticus* (Vp), *V. cholerae* (Vc) and *V. vulnificus* (Vv) are an established cause of Foodborne related illness. Worldwide these bacteria represent an important public health risk associated with seafood consumption. This interlaboratory study (ILS) was designed to support methodological improvements for the identification of *Vibrio* spp. using Polymerase chain reaction (PCR) and real-time PCR (RT-PCR) formats. It was noted at the most recent NRL network meeting (16th) in May 2017 the need for the development of quantitative methods for the determination of Vp in seafood safety; this ILS was subsequently initiated to help methodological development in this area.

Samples

Sample preparation

Thirty individual strains were selected from the Cefas *Vibrio* spp. reference strain bank and/or donated from other NRLs (Table 1). Strains were chosen to represent clinically relevant isolates as well as isolates that could challenge species-specific and pathogenicity-based PCR assays. Each strain was streaked onto a non-selective agar plate (Marine agar (MA)) and a selective agar plate (Tryptone citrate bile salts agar (TCBS)) and incubated for 18-24 hrs at 30±2 °C and 37±2 °C respectively.

Following visual purity checks, PCR was performed on each selected strain to confirm strain identification and pathogenic markers according to Annex C and D of ISO 21872-1. Each strain was allocated a designated sample code (Sample 1 – 30). Between 2 - 5 colony forming units (cfu) were isolated from the MA plate and inoculated into 30 ml of alkaline salt peptone water (ASPW) and incubated for 18-24 hrs at 30±2 °C. Following incubation, 1 ml aliquots were dispensed into 1.5 ml Eppendorf tubes and boiled at 95±2 °C for 5 mins to release the nucleic acids. Samples were then stored at >-15 °C until required.

Sample distribution

Samples were dispatched in accordance with IATA packing instructions 650 for UN3373 'Diagnostic Specimens' on the 10th July 2017 to 13 participating laboratories. Non-Vp strains were also included in this ILS to challenge species specificity of Vp identification methods. On receipt, participants were requested to examine the samples using their routine molecular method (PCR or RT-PCR) for the detection of Vp, Vv and Vc and where required, the pathogenic markers.

Results

Reference results

Reference analyses were performed on 3 replicates for each sample distributed using a combination of conventional and RT-PCR according to Annex C and D of ISO 21872-1. Reference results are summarised in Table 1.

Participants methodology

From the information provided by participants, 6 used conventional PCR, 2 used RT-PCR and 5 used a combination of both conventional and RT-PCR. Table 2 shows the references used to obtain the primer and probe sets (see key to indicate published reference).

Table 1: Reference results for PT 71 proficiency testing material

Sample No.	Strain type	Environmental / Clinical / Reference	Location	*ToxR*	*tdh*	*trh*	*VVH*
1	*V. parahaemolyticus*	Reference	UK	-	*d*	-	-
2	*V. parahaemolyticus*	Clinical	Norway	+	+	-	-
3	*S. Nottingham*	Reference	UK	-	-	-	-
4	*V. parahaemolyticus*	Clinical	Norway	+	-	-	-
5	*E. faecalis*	Reference	UK	-	-	-	-
6	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
7	*V. parahaemolyticus*	Reference	UK	+	+	-	-
8	*V. parahaemolyticus*	Clinical	UK	+	+	+	-
9	*V. parahaemolyticus*	Reference	UK	+	+	-	-
10	*V. parahaemolyticus*	Clinical	Norway	+	+	-	-
11	*V. alginolyticus*	Environmental	UK	-	-	-	-
12	*V. parahaemolyticus*	Clinical	Norway	+	+	-	-
13	*V. parahaemolyticus*	Clinical	Italy	+	+	-	-
14	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
15	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
16	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
17	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
18	*V. parahaemolyticus*	Clinical	Spain	+	+	-	-
19	*V. parahaemolyticus*	Clinical	Norway	+	+	-	-
20	*V. vulnificus*	Reference	UK	-	-	-	+
21	*V. parahaemolyticus*	Environmental	UK	+	-	*d*	-
22	*E. coli*	Reference	UK	-	-	-	-
23	*V. parahaemolyticus*	Environmental	Portugal	+	-	+	-
24	*V. parahaemolyticus*	Environmental	Portugal	+	-	+	-
25	*V. parahaemolyticus*	Clinical	Norway	+	+	+	-
26	*V. parahaemolyticus*	Clinical	Norway	+	-	*d*	-
27	*V. parahaemolyticus*	Clinical	Norway	+	+	*d*	-
28	*V. parahaemolyticus*	Clinical	Spain	+	+	+	-
29	*V. parahaemolyticus*	Clinical	Spain	+	+	+	-
30	*V. parahaemolyticus*	CIP Reference strain	France	+	-	*d*	-

d ≤ 66% gave negative results

Table 2: Participants primer and probe references

ID	Conventional PCR						Real-time PCR					
	ToxR	*TLH*	*TDH*	*TRH*	*vvhA*	*VC*	*ToxR*	*TLH*	*TDH*	*TRH*	*vvhA*	*VC*
7 *	N	B	B	B	J	G			T	T		
10	N		B	B	J	G						
21	A	B	B									
32	A		W	W	A	Z	X	H				
35				B		G	X		T		F	
39	N	Y	S	K	J	G						
41	O		S	K, L	P	G	I0		I	I	M	V
42	N		U	U	J	G						
68	N	B	B, W	B, W	J	G	X		T		F	
147	N	B	B	E	J	G						
167	O		B	R	D	G						
209 **							X		T	AA	F	Q
222							C	C	C	C	C	C

Key: A - Bauer and Rorvik 2007; **B** - Bej *et al.* 1999; **C** - Biotecon; **D** - Brauns *et al.* 1991; **E** - Cabrera-Garcia *et al.* 2004; **F** - Campbell *et al.* 2003; **G** - Chun *et al.* 1999; **H** - Eschbach *et al.* 2017; **I** - Hervio-Heath *et al.*; **J** - Hill *et al.* 1991; **K** - Honda and Lida 1993; **L** - Honda *et al.* 1991; **M** - Jones *et al.* 2012; **N** - Kim *et al.* 1999; **O** - Lee *et al.* 1995; **P** - Lee *et al.* 1997; **Q** - Lyon *et al.* 2001; **R** - Nilsson and Turner 2016; **S** - Nishibuchi and Kaper 1985; **T** - Nordstrom *et al.* 2007; **U** - Paterson *et al.* 1999; **V** - Robert-Pillot *et al.* 2014; **W** - Tada *et al.* 1992; **X** - Taiwo *et al.* 2017; **Y** – Taniguchi *et al.* 1985; **Z** - Tarr *et al.* 2007; **AA** – Ward and Bej 2006.

* Real-time PCR was used for samples that gave questionable result but has not been included in the full report.
** *V. cholerae* forward primer and probe based on Lyon *et al.* reference. The reverse primer used in the ILS was designed in-house.

Discussion

Thirteen laboratories (10 NRLs and 3 Non-NRLs) participated in this ILS (PT 71). Participants used a variety of species-specific PCR methods for the detection of vibrios distributed by the EURL (Table 3). These included 8 different approaches (mostly published), with 4 assays targeting the marker *toxR* (Kim *et al.* (1999), Bauer and Rorvik (2007), Taiwo *et al*. (2017) and Hervio-Heath (2017, unpublished)), *tlh* (Bej *et al.* (1999), Eschbach *et al.* (2017), Taniguchi (1985)) and *r72h* (Lee *et al.* (1995)). The most popular species-specific determination method was Kim *et al.* (1999). False-negative Vp identification was common across all strains, with 23 % of reported results lacked concordance with the EURL assigned identification using Kim *et al.* (1999) and 28 % using Lee *et al.* (1995). Both the Bauer and Rorvik (2007) and Taiwo *et al*. (2017) methods did not generate false-negative results in this ILS (Table 3). However, both the Bauer and Rorvik (2007) and Taiwo *et al.* (2017) methods gave the worst performance for false-positives results (17 %) compared with other PCR methods. The generation of false-positive results appeared to be less widespread with several methods performing well, including the Taniguchi (1985), Hervio-Heath (2017) and Eschback *et al.* (2017), where no false-positives were reported.

Taken together, the best performing PCR methods giving the best concordance with the EURL assigned identification from this ILS was the Bauer and Rorvik (2007), Hervio-Heath (2017) and Taniguchi (1985). However, the observation that all methods generated 5 % false-negative results should be noted and require further characterisation. It should be noted that the Bauer and Rorvik (2007) and Taniguchi (1985) methods are based on conventional PCR, whilst the best performing RT-PCR method was the Hervio-Heath (2017) and Eschbach *et al.* (2017) assays that targets *toxR* and *tlh* respectively.

The haemolysin genes *tdh* and *trh* are considered the most important markers associated with human virulence and are well-established targets used to determine pathogenic and non-pathogenic strains of Vp. A comparison of data generated from both *tdh* and *trh* PCR methods provided an important insight into the reliability of these testing assays.

Table 3. Data generated from *V. parahaemolyticus* species-specific markers

Reference	PCR method	No. of labs	ToxR / TLH	False +ve	False -ve
Bauer and Rorvik (2007)	Conventional	2	*ToxR*	17 %	0 %
Kim *et al*. (1999)	Conventional	6	*ToxR*	3 %	23 %
Bej *et al*. (1999)	Conventional	4	*TLH*	4 %	16 %
Taniguchi (1985)	Conventional	1	*TLH*	0 %	5 %
Lee *et al*. (1995)	Conventional	2	*r72h*	0 %	28 %
Taiwo *et al.* (2017)	real-time	4	*ToxR*	17 %	0 %
Hervio-Heath *et al.* (2017, unpublished)	real-time	1	*ToxR*	0 %	5 %
Eschbach *et al.* (2017)	real-time	1	*TLH*	0 %	5 %

Seven different PCR assays for *tdh* were used by participants, which encompassed many of the well-established and published PCR assays for this target. The most popular and widely used *tdh* PCR assay is Bej *et al.* (1999) with 6 laboratories using this method (Table 4) and was one of the best performing conventional PCR methods during this ILS, with 12.5 % false-positive and 6 % false-negative results reported in concordance with the EURL assigned identifications. Both the Tada *et al.* (1992) and Nishibuchi and Kaper (1985) PCR assays provided excellent discrimination of strains, but suffered from false-positive results (Table 4). The best performing RT-PCR method was Nordstrom *et al.* (2007), which generated 5 % false-positive and 6 % false-negative results during this ILS. It should be noted that the Bej *et al.* (1999) method is based on conventional PCR.

Table 4. Data generated from *V. parahaemolyticus tdh* test distribution

Reference	PCR method	No. of labs	*False +ve*	*False -ve*
Bej *et al.* (1999)	Conventional	6	12.5 %	6 %
Tada *et al.* (1992)	Conventional	2	0 %	17 %
Nishibuchi and Kaper (1985)	Conventional	2	0 %	19 %
Paterson *et al.* (1999)	Conventional	1	0 %	61 %
Nordstrom *et al.* (2007)	real-time	3	4 %	6 %
Hervio-Heath *et al.* (2017, unpublished)	real-time	1	13 %	6 %
Biotecon	real-time	1	75 %	0 %

Analysis of *trh* provided interesting insights into the performance of this assay across a range of different laboratories. Nine different PCR assays were utilised here, again representing a diverse overview of published PCR assays used internationally for this target gene. A general observation, and a finding that we can corroborate is that conventional PCR tended to perform more effectively than RT-PCR (Table 5). The best performing assay (and used by two different laboratories) was the Nilsson and Turner (2016) PCR assay, that generated 100 % concordance with the EURL assigned identification. The Paterson PCR method, which was used by just one laboratory, generated extremely high false-negative results (83 %). The Ward and Bej (2006) RT-PCR assay generated extremely good detection characteristics during this ILS (with 0 % false-negative and 5 % false-positive) and as such represented the best performing RT-PCR assay observed here. It should be noted however, that these results were generated from one laboratory only. The EURL has also experienced significant issues using this RT-PCR assay (as with other RT-PCR assays for *trh*) and tend to use conventional PCR assays to detect the *trh* gene in routine analysis. The Biotecon PCR assay also performed well, although 10 % of strains generated false-positive results in this ILS.

Table 5. Data generated from *V. parahaemolyticus trh* test distribution

Reference	PCR method	No. of labs	*False +ve*	*False -ve*
Bej *et al.* (1999)	Conventional	4	0 %	4 %
Tada *et al.* (1992)	Conventional	2	3 %	0 %
Nilsson and Turner (2016)	Conventional	2	0 %	0 %
Honda and Lida (1993)	Conventional	2	0 %	42 %
Paterson *et al.* (1999)	Conventional	1	0 %	83 %
Cabrera-Garcia *et al.* (2004)	Conventional	1	10 %	17 %
Ward and Bej (2007)	real-time	1	5 %	0 %
Hervio-Heath *et al.* (2017, unpublished)	real-time	2	5 %	42 %
Biotecon	real-time	1	10 %	0 %

Conclusions

This ILS exercise has been important in allowing a direct comparison of different conventional and RT-PCR assays used across a large number of laboratories, using Vp as the focus organism. The aim of this ILS was to facilitate method development rather than laboratory performance, in particular for PCR methodologies focussed on the pathogen Vp. Some notable issues and conclusions were identified during this ILS, including:

- Anomalous/inconsistent PCR results being recorded in a small number of reference samples. For future ILS and PT exercise the EURL will attempt to use (and where possible) fully genomically sequenced strains, as this provides additional information to unambiguously characterise bacterial isolates.
- A number of species-specific assays were assessed as part of this ILS, and these vary in terms of performance (e.g. generation of false-positive and false-negative results).
- The best performing RT-PCR methods observed during this ILS was Hervio-Heath (2017) and Eschbach *et al.* (2017) that target *toxR* and *tlh*, respectively. These assays should be assessed in more detail to determine their overall utility for future comprehensive ILS and PT exercises. An area of work that could be undertaken under the responsibility of the EURL would be the generation of primer and probes for these two assays that could be circulated to laboratories interested in participating in the next PT exercise.
- For the haemolysin genes *tdh* and *trh,* in this ILS exercise it was noted that the Nordstrom *et al.* (2007) and Ward and Bej (2006) assays performed best (respectively) and could be the focus of future ILS and PT exercises, in particular using a wide range of *trh1* and *trh2* strains that have undergone whole genome sequencing to determine the overall utility of this method.

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Head office
Centre for Environment,
Fisheries & Aquaculture Science
Pakefield Road, Lowestoft,
Suffolk NR33 0HT UK

Tel +44 (0) 1502 56 2244
Fax +44 (0) 1502 51 3865
Web www.cefas.defra.gov.uk

Centre for Environment,
Fisheries & Aquaculture Science
Weymouth Laboratory,
Barrack Road, The Nothe, Weymouth,
Dorset DT4 8UB

Tel +44 (0) 1305 206600
Fax +44 (0) 1305 206601

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