Centre for Environment Fisheries & Aquaculture Science

Cefas

European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

EURL method comparison of *vvHA V. vulnificus* real-time PCR assay (December 2016).

1. Introduction

A PCR method described in ISO 21872 for the detection and confirmation of *V. vulnificus* was recently identified to contain an erroneous reverse primer sequence. Specifically, the reverse primer for the *vvHA* real-time PCR omitted a guanine nucleotide residue at the final position (see below, indicated in red):

VVHA (FW):	TGT TTA TGG TGA GAA CGG TGA CA[1]
VVHA (REV):	TTC TTT ATC TAG GCC CCA AAC TTG[1]
VVHA (PROBE):	CCG TTA ACC GAA CCA CCC GCA A[1]

Probes were labelled 5' 6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA)

We believe that this was a transcription error introduced erroneously during the selection of primers and probe sequences during earlier work by the EURL. Unfortunately, this incorrect sequence was included as a set of primers and sequences used during the interlaboratory study undertaken during the establishment of this ISO. Because of the potential for this error to have had impacts on the validity of this real-time PCR assay, the EURL undertook both desk-based and practical studies to determine the impact, if any, on the results generated with these two primer sequences.

2. Desk-based study

When we became aware of this sequence error the EURL carried out a brief analysis of: 1) Results obtained from other laboratories during the inter-laboratory study (ILS), 2) Results generated in house using these primer sets, and 3) *In silico* data regarding the potential impact of this error. A review of data obtained from the ILS (where the erroneous sequence was supplied to several laboratories for use) indicated that a geographically diverse set of participating laboratories had used this incorrect reverse sequence successfully to qualitatively detect *V. vulnificus.* This finding was cross-checked against the primer sequences ordered and supplied to the laboratory to confirm. This information suggests that this did not affect the performance of this assay as a means of identifying *V. vulnificus.* We also reviewed data carried out at the EURL for the detection of *V. vulnificus* using both strains that have been obtained from both seafood-derived as well as clinical sources. This review of data (and where the primer and probe sequences could be effectively cross-checked) also indicates no obvious deleterious impact of this incorrect reverse primer sequence on the ability of the *vvHA* PCR to identify *V. vulnificus.* A further analysis of the implicated DNA sequence was performed. This was carried out to determine if the region targeted by the reverse real-time PCR primer could

have been impacted by the omission of a single nucleotide at the final position. The EURL downloaded sequence data from publicly available databases (NCBI) and carried out a sequence similarity search using BLAST of 27 strains of *V. vulnificus.* This sequence data was then used to derive a nucleotide alignment of the relevant fragment of the *vvHA* gene. The position of the reverse primer sequence can be observed in black (nucleotide positions 77-100). The missing nucleotide position (position 77, red arrow). The gene is highly conserved in the target region, with only one polymorphism identified anywhere (and only in one strain, position 28 in strain 628-7), and none whatsoever in the primer binding regions. The nucleotide in question is the C at position 77 (binds with the G of the reverse primer). Based on this we determine that although the mistake in the primer sequence could conceivably impact the PCR efficiency to some extent (by reducing the melt temperature of the reverse primer), the probability that this would prevent the PCR from effective amplification is highly unlikely. The melting temperature (T_m) of the primer with and without the guanine residue is 54 ^oC and 51.7 ^oC, respectively.

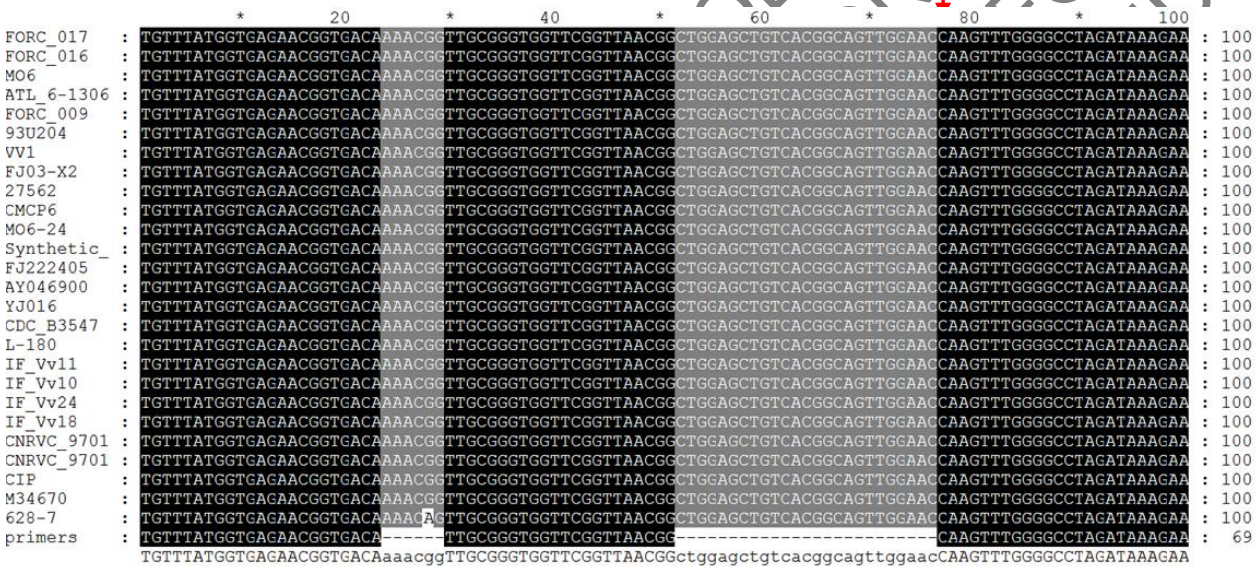

Figure. 1. Nucleotide alignment of *vvHA* sequences derived from publicly-available databases with the relevant primer and probe sequences (indicated in black). The reverse primer site is also indicated (black region, starting with the red arrow positions 78-100). The position on the missing guanine residue is indicated by the red arrow (position 77).

3. Laboratory-based verification

To further ascertain the potential impact of the missing nucleotide on the *vvHA* real-time PCR assay the EURL carried out a short verification study. Briefly, the *vvHA* primer and probe sequences were ordered and included two sets of separate reverse primer (e.g. those including and omitting the missing guanine residue):

VVHA1 (REV): TTC TTT ATC TAG GCC CCA AAC TTG[1]

VVHA2 (REV): TTC TTT ATC TAG GCC CCA AAC TT[1]

A side-by-side comparison of the assay was subsequently undertaken using both primer sets. *V. vulnificus* strains (20 isolates), encompassing clinical and environmental isolates were

firstly tested using conventional PCR to confirm identities[2]. Positively identified *V. vulnificus* strains were then boiled in 200 µl molecular grade water and subsequently cooled in ice prior to testing. A total of 37 bacterial strains, including *V. vulnificus* (*n* = 20), other *Vibrio* species (*n* = 11), and distantly related reference strains (*n* = 6) were used to assess the specificity of the oligonucleotide probe and primer sets used (Annex A). For real-time PCR experiments, the assay comprised of a total reaction of 25 µl, consisting of 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.45 µl each of forward and reverse primer (as appropriate), *V. vulnificus vvHA* primers (100 nM), 5.6 µl nuclease-free water, and 1 µl of probe (500 nM). Five microliters of template (boiled cell lysate) was subsequently added, and each reaction was performed in duplicate. Real-time PCR was carried out using the two primer sets (e.g. *vvHA1* and *vvHA2*) on each individual boiled cell homogenate. Congruence was determined as either a positive or negative result indicated by the presence or absence of a sigmoidal amplification curve. A comparison between analysed primer sets subsequently identified complete congruence between assays, with both *vvHA1* and *vvHA2* demonstrating complete agreement between tested homogenates. Indeed, the Cycle Threshold (C_t) data between both assays is strikingly similar and in most instances, is within 1 C_t value (Table 1). No amplification of closely related *Vibrio* or non-*Vibrio* strains were observed, indicating the specificity of the *vvHA* real-time PCR assay between both tested assay sets. A visual inspection of the real-time PCR results from this study also showed that amplification curves between *vvHA1* and *vvHA2* were identical (Figure 2). This data is important because it demonstrated no significant impact of the missing nucleotide residue on the efficiency of the PCR assay, which is outlined in ISO 21872 as a qualitative test method (e.g. presence/absence).

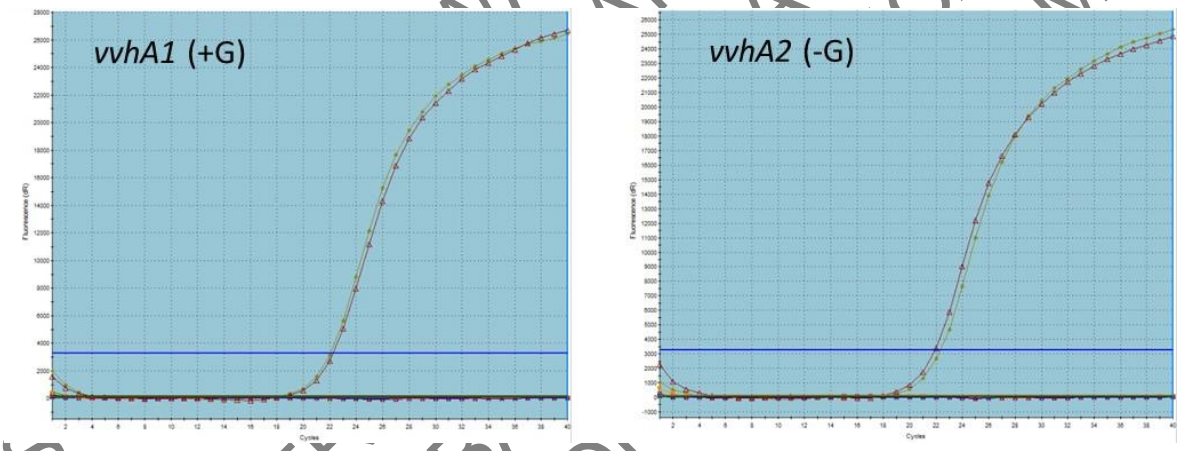

Figure. 2. Side-by-side comparison of two real-time PCR tests (strain 14, Annex A) demonstrating comparable amplification curves between samples tested with vvhA1 (with guanine, left) and vvhA2 primer (without guanine, right) residue. Assays demonstrate expected sigmoidal amplification curves (both within less than a single C_t value).

Conclusions

Our findings indicate that the error regarding the missing guanine residue in the primer sets used in both the ILS as well as a qualitative test method to identify *V. vulnificus* would not detrimentally affect the performance of this outlined PCR test. This finding is based on data obtained from the ILS, our own practical use of the assay, an *in silico* analysis of the assay binding region but also critically from a side-by-side verification of both PCR assays carried out in house. Taken together this data indicates that these two assays are identical in their ability to positively identify *V. vulnificus.*

Annex A

Strain tested	Average C_t *vvhA1*	Average C_t *vvhA2*
V. vulnificus 10	24.3	24.4
V. vulnificus 6	22.6	21.7
V. vulnificus 7	22.6	22.3
V. vulnificus 38	25.1	24.9
V. vulnificus 19	21.7	21.5
V. vulnificus 1	22.1	22.6*
V. vulnificus 32	22.5	22.2
V. vulnificus 11	22	21.9
V. vulnificus 16	22.6	22.6
V. vulnificus 18	22.5	22.1
V. vulnificus 42	35.2	35.3
V. vulnificus 5	23.2	22.7
V. vulnificus 33	21.8	21.6
V. vulnificus 30	20.1	19.7
V. vulnificus 14	22.2	22.1
V. vulnificus 29	19.1	18.6
V. vulnificus 36	20.9	20.4
V. vulnificus 8	23.1	23.5
V. vulnificus 9	20.3	20.8
V. vulnificus 24	22	20.5
V. parahaemolyticus (*n*=6)	ND	ND
V. cholerae (*n*=4)	ND	ND
V. alginolyticus (*n*=1)	ND	ND
E. faecialis (NCTC 775)	ND	ND
E. coli (NCTC 12241)	ND	ND
P. aeruginosa (NCTC 10332)	ND	ND
E. coli (13216)	ND	ND
Salmonella Nottingham (NCTC 7832)	ND	ND
P. mirabilis (NCTC 10975)	ND	ND

*1 from 2 results were positive. Data based on single positive result.

References

1. CAMPBELL M.S., WRIGHT A.C. Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Appl. Environ. Microbiol*. 2003, 69 pp. 7137–7144.
2. HILL W.E., KEASLER S.P., TRUCKSESS M.W., FENG P., KAYSNER C.A., LAMPEL K.A. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl. Environ. Microbiol*. 1991, 57 pp. 707–711.