Validation of real-time PCR for detection of six major pathogens in seafood products

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Abstract

Seafood can pose a public health concern to consumers. It is often consumed raw and may be contaminated with several foodborne pathogens. In order to guarantee the safety of seafood, real-time polymerase chain reaction (PCR) protocols may be used as these enable results to be provided within 24 h.

The first goal of our work was to develop real-time PCR protocols enabling the detection of six foodborne pathogens that may be present in seafood products (Campylobacter coli, Campylobacter jejuni, Escherichia coli, Salmonella spp., Vibrio parahaemolyticus, and Vibrio vulniﬁcus). The corresponding gene targets were: 5OS/VS1, rfbE, ttr, tlh, and vvp. A multiplex PCR was also developed to detect the virulence genes of V. parahaemolyticus: tdh and trh. A total of 420 bacterial strains belonging to four different genera/strains were used in this study. Sensitivity and specificity were always 100%, except in the case of Salmonella spp., where three strains were not detected by our PCR protocols.

The second objective of our work was to assess the detection limit of our real-time PCR protocols on artiﬁcially contaminated seafood products (raw shrimps, cooked shrimps, and raw mussels), purchased in public stores. Six different levels of contamination were assayed in four replicates for each matrix. The real-time PCR protocols enabled a better level of detection than the ISO methods, except for Salmonella in raw shrimps and for V. vulniﬁcus in shrimps (raw and cooked). The estimated level of detection was between 1 and 47 cfu/25 g sample for the ISO norms and between 1 and 315 cfu/25 g sample for the real-time PCR protocols tailored in our work.

The real-time PCRs developed in our work allowed for good selectivity, sensitivity, and speciﬁcity. The sensitivity on seafood products was estimated at a level of 100%, except for Salmonella (97%). In the spiking assays, the levels of detection were lower with the real-time PCR protocol than those obtained with the ISO method. This was not the case for V. vulniﬁcus in raw and cooked shrimps and for Salmonella in raw shrimps.

These real-time PCR protocols appear to be good alternative methods for surveillance of seafood products to ensure the absence of foodborne pathogens.

One additional conclusion is that laboratories have to use enrichment media that are compatible with those recommended by ISO standards. This may facilitate the isolation of the pathogen if the real-time PCR protocol gives a suspect positive signal during the first step of the seafood analysis.

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1. Introduction

Shellﬁsh and crustaceans play an important role in foodborne diseases all over the world, including in developed countries. They can become contaminated by the environment owing to the fact that bivalve mollusks ﬁlter a signiﬁcant volume of water. The latter explains the concentration of the pollutants in the tissues of mollusks. Additional reasons can be related to the pollution of water by human feces, the contamination of cropping areas due to ﬂooding, the contamination of crops by agricultural activities, and the importation of shellﬁsh from foreign countries that do not apply good and reliable surveillance methods (Iwamoto, Ayers, Mahon, & Swerdlow, 2010). In developed countries, most infections result from the ingestion of raw or undercooked seafood (Chen et al., 2012). Our gastronomic culture favors raw seafood, facilitating the persistence and transmission of possible microbial contaminants. Moreover, the consumption of seafood is still increasing and represents a worldwide market. Following Iwamoto

Abbreviations: BHI, Brain Heart Infusion; cfu, colonies forming units; ISO, International Organization for Standardization; PCR, Polymerase Chain Reaction.

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et al. (2010), the consumption of seafood in the USA has risen by 60% on average between the 1980s and the present decade (from 4.5 to 7.2 kg/year/inhabitant).

In Europe, 8.5% of foodborne outbreaks (i.e. 59 out of a total of 698) have been related to crustaceans, shellfish, mollusks, and products thereof (European Food Safety Authority & European Commission, 2005). There is the possibility of using alternative reliable identification tools for seafood control.

In Europe, on the basis of Regulation (EC) No 2073/2005, conventional analytical methods have to be used (European Commission, 2005). There is the possibility of using alternative methods, if they are validated by official authorities. One of the major breakthroughs is the use of real-time quantitative polymerase chain reaction (qPCR), making this technology available at an affordable price. Following Boyer and Combrisson (2013) qPCR has multiple advantages: the detection of Listeria monocytogenes can take 2 days with qPCR and it can also overcome the problem of viable but non-culturable microorganisms (VBNC). In seafood, several studies have been carried out, for example: simultaneous detection of Salmonella and Listeria in salmon samples (Amagliani, Omiccioli, Brandi, Bruce, & Magnani, 2010), study of the persistence of V. vulnificus in surface water (Randa, Polz, & Lim, 2004), and the use of TaqMan qPCR to detect V. cholerae in raw oysters (Lyon, 2001).

Our objectives were to present alternative qPCR methods developed and validated in parallel with the International Organization for Standardization (ISO) methods. We chose to focus our study on pathogens for which validated standard protocols are available: enterohemorrhagic Escherichia coli, Salmonella spp, V. parahaemolyticus, V. cholerae, V. vulnificus, Campylobacter jejuni, and Campylobacter coli. The aims of our study were as follows: first, to assess the sensitivity and specificity of real-time qPCR on our collection of bacterial strains and, second, to perform a validation for pathogen detection in shrimp and mussel samples.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and media

A total of 420 bacterial strains were used in this study. All bacterial strains came from our laboratory culture collection (University of Liège) kindly provided by different organizations (BCCM, Institut Pasteur of Paris etc.) or isolated from contaminated food or hospitalized patients suffering from foodborne diseases (Table 1). Except for V. parahaemolyticus and Campylobacter spp., all isolates were routinely grown overnight in Tryptone Soy Broth (TSB) and in Tryptone Soy Agar (TSA) at 37 °C (Oxoid, Aalst, Belgium). V. parahaemolyticus isolates were grown in 2% Alkaline Peptone Water (APW) at 37 °C for 24 h, while Campylobacter isolates were grown in Brucella broth under microaerophilic conditions, using the same incubation conditions.

For the spiking assays in seafood products, the following reference strains were used: E. coli O157:H7 — ATCC 43890; Salmonella — ATCC 13076; V. parahaemolyticus — ATCC 43996; V. vulnificus — ATCC 27562; C. jejuni — ATCC 33560; C. coli — strain 304 (kindly provided by Prof. de Zutter, Gent University).

2.2. Real-time PCR protocols

All primers and probes were designed specifically for this study with the exception of the real-time PCR for the detection of Salmonella spp. In the latter, the study of Malorny et al. (2004) was taken into account when designing the primers (Tables 2 and 3). The primers and probes were designed using Primer3 (Rozen & Skaltsky, 1999) from alignments made with CLUSTALW shareware (available online at http://www.ebi.ac.uk/Tools/msa/clustalo/ ) of the various target sequences present in GenBank. The specificity of the primers and probes was first checked in silico with the most homologous sequences to the target gene from neighboring

Table 1

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter coli</td>
<td>13</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>27</td>
</tr>
<tr>
<td>Campylobacter spp.</td>
<td>7</td>
</tr>
<tr>
<td>Escherichia coli O157:H7</td>
<td>35</td>
</tr>
<tr>
<td>Escherichia coli non-O157:H7</td>
<td>62</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>136</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>35</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>114</td>
</tr>
<tr>
<td>Vibrio spp.</td>
<td>101</td>
</tr>
<tr>
<td>Total</td>
<td>420</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene target</th>
<th>Designation</th>
<th>Sequence 5’-3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni and</td>
<td>50S</td>
<td>50S-F</td>
<td>GCGGGTTCACAAAGCAGACATACG</td>
<td>This study</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>50S-R</td>
<td>[VIC]-TGCTGTCGGCGCTGGCTGCC-TAMRA-[NFQ-MGB]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50S-probe</td>
<td>[VIC]-TGCTGTCGGCGCTGGCTGCC-TAMRA-[NFQ-MGB]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V5I-F</td>
<td>GTATGTCTTCTATTGTTGACCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V5I-R</td>
<td>GTATGTCTTCTATTGTTGACCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V5I-probe</td>
<td>CCGGTTCACAAAGCAGACATACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli O157:H7</td>
<td>rfbE-F</td>
<td>AAGAATCCACCTGGACATAAACCTC</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rfbE-R</td>
<td>TGCAGGTACATTTGCGATCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rfbE-probe</td>
<td>[FAM]-CGAACAAACCGTTTGCATACCGT-TAMRA-[NFQ-MGB]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>ttr-F</td>
<td>CTCACGAGGATCATACCGGATG</td>
<td>(Malorny et al., 2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ttr-R</td>
<td>AGTCTCAACGACAAACACTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ttr-probe</td>
<td>[FAM]-CACCGACGACGACGATTT-[TAMRA]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FAM, VIC, YY: fluorophores. BHQ1, TAMRA, NFQ: quenchers. NFQ-MGB: DNA-minor groove binding moiety.
bacterial species. The primers and probes were synthesized by Sigma Genomic (Diegem, Belgium).

Total bacterial DNA was extracted from exponential phase grown cultures with the Wizard® Genomic DNA Purification Kit (Promega, Leiden, The Netherlands), according to the manufacturer’s protocols. For Gram-positive bacteria, lysozyme (at a concentration of 10 mg/ml) was added to the lysis solution (following manufacturer’s instructions). DNA was solubilized in water and stored at 4 °C.

Three different protocols were investigated and optimized for simplex or multiplex use (Tables 4 and 5). The following genes were targeted: rfbE gene, implicated in the O157 somatic antigen synthesis in enterohemorrhagic E. coli (Guan et al., 2013; Suo, He, Tu, & Shi, 2010); tdh, which is the thermostable direct hemolysin gene in V. parahaemolyticus (Robert-Pillot et al., 2004); ttr gene, required for tetraethionate respiration and located in a highly conserved area of the Salmonella genome (Malorny et al., 2004); and the vvp gene coding for a metalloprotease and used for specific detection of V. vulnificus. The amount of enzyme and master mix was added in order to reach a total reaction volume of 25 µl following the manufacturer’s protocol. The amplifications were carried out as follows: denaturation at 95 °C for 10 min followed by 40 cycles of denaturation (95 °C for 15 s) and annealing at variable temperatures for 60 s. All details regarding amplification and annealing temperatures are presented in Table 5. All protocols were operated on an ABI 7900 device (Applied Biosystem, USA).

When needed, an initial step of polymerase activation (amperase activity) was carried out at 50 °C for 30 min before the

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene target</th>
<th>Designation</th>
<th>Sequence 5'–3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tth</td>
<td>tth-F</td>
<td>GAAAGCGGCTCCAGTTTAAG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tth-R</td>
<td>ACGTGATCTTCACGGCCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[FAM]-CCGAGAGGATCCCATGCGG- TMARA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tdh</td>
<td>tdh-F</td>
<td>GCAGGGGTGTCTGGCGTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tdh-R</td>
<td>AGGAACTGGAAGTCTGACTCTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tdh-probe</td>
<td>[FAM]-CGGCTTCTGTCCTGCTGTA- [BHQ1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>vvp</td>
<td>vvp-F</td>
<td>TCTCGGTCTCATGCTTGTGCA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vvp-R</td>
<td>TCGGACAGGGACACATTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vvp-probe</td>
<td>FAM]-CATCGGTCTTCTCAGCTCAGCGTGC-[TMARA]</td>
<td></td>
</tr>
</tbody>
</table>

FAM, VIC, YY: fluorophores. BHQ1, TAMRA, NFQ: quenchers. NFQ-MGB: DNA-minor groove binding moiety.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Concentration of primer(s) F used</th>
<th>Concentration of primer(s) R used</th>
<th>Probe(s)</th>
<th>Enzyme†</th>
<th>Final DNA concentration</th>
<th>Targeted genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTPCR-1</td>
<td>50 µM</td>
<td>50 µM</td>
<td>20 µM</td>
<td>Taqman</td>
<td>10–100 ng</td>
<td>rfbE, ttr, &amp; vvp</td>
</tr>
<tr>
<td>RTPCR-2</td>
<td>50 µM</td>
<td>50 µM</td>
<td>20 µM</td>
<td>iTaq</td>
<td>10–100 ng</td>
<td>tth</td>
</tr>
<tr>
<td>RTPCRM-1</td>
<td>50 µM/50 µM</td>
<td>50 µM</td>
<td>20 µM/20 µM</td>
<td>Taqman</td>
<td>10–100 ng</td>
<td>tdh/trh &amp; 50S/VS1</td>
</tr>
</tbody>
</table>

† Taqman — FG, Taqman Universal PCR MSTR Mix, Amperase UNG (Applied Biosystem, Carlsbad, USA). iTaq — iTaq Supermix with Rox (Biorad).

### Table 3

Real-time PCR primers and probe sequences used for simplex and multiplex detection for Vibrio parahaemolyticus and Vibrio vulnificus.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene target</th>
<th>Designation</th>
<th>Sequence 5'–3'</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tth</td>
<td>tth-F</td>
<td>GAAAGCGGCTCCAGTTTAAG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tth-R</td>
<td>ACGTGATCTTCACGGCCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[FAM]-CCGAGAGGATCCCATGCGG- TMARA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>vvp</td>
<td>vvp-F</td>
<td>TCTCGGTCTCATGCTTGTGCA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vvp-R</td>
<td>TCGGACAGGGACACATTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>vvp-probe</td>
<td>FAM]-CATCGGTCTTCTCAGCTCAGCGTGC-[TMARA]</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4

Enzymes and concentrations of primers and probes used for the real-time PCR protocols.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Concentration of primer(s) F used</th>
<th>Concentration of primer(s) R used</th>
<th>Probe(s)</th>
<th>Enzyme†</th>
<th>Final DNA concentration</th>
<th>Targeted genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTPCR-1</td>
<td>50 µM</td>
<td>50 µM</td>
<td>20 µM</td>
<td>Taqman</td>
<td>10–100 ng</td>
<td>rfbE, ttr, &amp; vvp</td>
</tr>
<tr>
<td>RTPCR-2</td>
<td>50 µM</td>
<td>50 µM</td>
<td>20 µM</td>
<td>iTaq</td>
<td>10–100 ng</td>
<td>tth</td>
</tr>
<tr>
<td>RTPCRM-1</td>
<td>50 µM/50 µM</td>
<td>50 µM</td>
<td>20 µM/20 µM</td>
<td>Taqman</td>
<td>10–100 ng</td>
<td>tdh/trh &amp; 50S/VS1</td>
</tr>
</tbody>
</table>

† Taqman — FG, Taqman Universal PCR MSTR Mix, Amperase UNG (Applied Biosystem, Carlsbad, USA). iTaq — iTaq Supermix with Rox (Biorad).

### Table 5

Conditions of PCR protocols of gene targets.

<table>
<thead>
<tr>
<th>Targeted genes</th>
<th>Purpose</th>
<th>PCR conditions</th>
<th>Pre-heating</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>rfbE</td>
<td>E. coli O157:H7 detection</td>
<td>50 °C—30 min</td>
<td>95 °C—10 min</td>
<td>95 °C—15 s</td>
</tr>
<tr>
<td>tdh</td>
<td>Virulence genes detection</td>
<td>50 °C—30 min</td>
<td>95 °C—10 min</td>
<td>95 °C—30 s</td>
</tr>
<tr>
<td>vvp</td>
<td>V. vulnificus detection</td>
<td>50 °C—30 min</td>
<td>95 °C—2 min</td>
<td>95 °C—30 s</td>
</tr>
<tr>
<td>ttr</td>
<td>Salmonella detection</td>
<td>50 °C—30 min</td>
<td>95 °C—2 min</td>
<td>95 °C—30 s</td>
</tr>
</tbody>
</table>
The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

Table 6
Validation results of the real-time PCR protocols for detection of foodborne pathogens.

<table>
<thead>
<tr>
<th>Test</th>
<th>Target gene</th>
<th>Target bacteria</th>
<th>Positives/n tested</th>
<th>Negatives/n tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>VS1/50S</td>
<td>C. jejuni</td>
<td>25/25</td>
<td>0/25</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>VS1/50S</td>
<td>C. coli</td>
<td>13/13</td>
<td>0/13</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>VS1/50S</td>
<td>Other Campylobacter</td>
<td>0/7</td>
<td>7/7</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>rfbE</td>
<td>E. coli O157:H7</td>
<td>26/26</td>
<td>0/26</td>
</tr>
<tr>
<td>Salmonella spp</td>
<td>trr</td>
<td>Salmonella spp.</td>
<td>97/100</td>
<td>3/100</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tih</td>
<td>V. parahaemolyticus</td>
<td>35/35</td>
<td>0/35</td>
</tr>
<tr>
<td>Vibrio vulnificus</td>
<td>vvp</td>
<td>V. vulnificus</td>
<td>4/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

2.4. Spiking assays on seafood products

Spiking assays were performed with three different seafood matrices: raw shrimps, cooked shrimps, and raw mussels. These seafood samples from four different batches were purchased in a local store in Liège, Belgium. Each matrix was inoculated with the following bacterial species: C. coli, C. jejuni, E. coli O157:H7, Salmonella spp., V. parahaemolyticus, and V. vulnificus, and at six different levels of contamination. Except in the cases of Vibrio and Campylobacter spp., isolates from reference strains were grown overnight in 2% BHI (Brain Heart Infusion) to reach the exponential growth phase. The spiking assay was designed to reach a bacterial load of approximately 0, 0.1, 1, 10, 100, and 1000 bacteria per 25 g of matrix. For the spiking assays, the same enrichment media described in Section 2.1 were used: TB0 or TSA for all the pathogens, except for V. parahaemolyticus (2% APW) and for Campylobacter (Brucella broth under microaerophilic conditions). Each seeded sample was performed in four replicates, with matrices taken from the four different batches. In order to assess the exact load of inoculated bacteria in each sample, the contamination broths at several dilutions were plated directly on corresponding non-selective media using a “spiral” device (AES Chemunex, France). Counting of bacteria was carried out after a specific incubation time for each pathogen following the recommendations of the ISO norms.

In order to compare the qPCR with classical methods, the targeted bacteria detection was performed with a validated microbiological ISO method according to the accepted protocol. These methods were respectively ISO 16654 (enterohemorrhagic E. coli), ISO 6579 Amendment 1 (Salmonella spp.), ISO 21872-1 (V. parahaemolyticus), ISO 21872-2 (V. vulnificus), and ISO 10272-1 (C. jejuni and C. coli). The qPCR protocols were performed on the same 24 h enrichment broth, following the spiking assay. The extraction of DNA was done using the protocol described earlier (see Section 2.3). The main objective of the spiking assay was to assess, on artificially contaminated seafood products, the detection limit of the real-time PCR in comparison with conventional bacteriological methods.

3. Results

3.1. Selectivity tests on pure cultures

Real-time PCR amplifications were validated with the bacterial collection from our laboratory. The strains from our collection were submitted to the real-time protocols to check the sensitivity of the method and to confirm the ability of the PCR protocol to detect the presence of virulence genes. For each validation trial, strains not belonging to the same group of bacteria were submitted to the same real-time PCR protocols.

Table 7
Validation results of the real-time PCR protocols for virulence detection of V. parahaemolyticus.

<table>
<thead>
<tr>
<th>Test</th>
<th>Target gene</th>
<th>Target bacteria</th>
<th>Positives/n tested</th>
<th>Negatives/n tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tih</td>
<td>V. p. tih+/trh+</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>tdr</td>
<td>V. p. tdr+/trh+</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>trh</td>
<td>V. p. trh+/trh+</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>vvp</td>
<td>V. p. vvp</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>vvp</td>
<td>V. p. vvp</td>
<td>0/11</td>
<td>11/11</td>
</tr>
</tbody>
</table>

Table 8
Campylobacter coli – Results of the spiking assay for the detection of Campylobacter coli in seafood samples.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Contamination level in 25 g sample (cfu/25 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deduced*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 0.2 0.3 1 2 3 6 28</td>
</tr>
<tr>
<td>Cooked shrimps</td>
<td>ISO 10272-1</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Real-time PCR</td>
<td>0/4</td>
</tr>
<tr>
<td>Raw shrimps</td>
<td>ISO 10272-1</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Real-time PCR</td>
<td>0/4</td>
</tr>
<tr>
<td>Raw mussels</td>
<td>ISO 10272-1</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Real-time PCR</td>
<td>0/4</td>
</tr>
</tbody>
</table>

The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

* Estimated value based on enumeration at higher level of contamination.
strains. Overall, the validation results were optimal and all the bacterial strains tested were well detected by our protocols. Moreover, no detection by the dedicated real-time PCR was obtained for all the isolates not belonging to the targeted group. In the case of V. parahaemolyticus, non-specific amplifications of 16S fragment were observed with other Vibrio species at high Cycle Threshold values (e.g. Ct of 26 for Vibrio campbellii, Ct of 30 for V. cholerae, and Ct of 33 for Vibrio alginolyticus). On the opposite, for V. parahaemolyticus, the Ct was always below 20 (data not shown). A result was thus considered positive when Ct values ranged between 7 and 20. Ct values higher than 20 were considered negative or unconfirmed results (data not shown).

3.2. Spiking assays on seafood products

The minimal detection limit was lower with real-time PCR than with the corresponding ISO method (Tables 8–13). For C. coli, the assessed contamination levels (in 25 g) ranged between 0.2 and 5700 cfu/25 g. No differences were observed except for the contamination level of 0.2 cfu/25 g in raw shrimps, where the corresponding ISO method detected three positive samples instead of four for real-time PCR method. With a contamination of 0.3 cfu/25 g, real-time PCR enabled the detection of four positive samples, while the ISO method did not detect any positive samples (Table 8).

In the case of C. jejuni (Table 9), real-time PCR was able to recover the four positive samples (5 cfu/25 g) in cooked shrimps, while; in contrast, the ISO method detected only two positive samples at this concentration. The difference in the level of detection was also observed with the contamination level of 1 cfu/25 g in raw mussels (four positive samples for real-time PCR and only one for the ISO method).

In relation to E. coli O157:H7 (Table 10), the real-time PCR technique detected four positive samples in all the tested matrices at an estimated level of contamination near 1 cfu/25 g of sample. At the same level of contamination, the ISO method was able to detect only two or three positive samples. When considering Salmonella spp. (Table 11), the results were not very conclusive, except for raw shrimps, where the ISO method seemed superior to real-time PCR for a contamination level of 2 and 24 cfu/25 g sample. In the case of V. vulnificus (Table 13), the ISO method was superior to the real-time PCR technique, especially for raw shrimps when considering the contamination levels of 3, 30, and 310 cfu/25 g (four positive samples for the ISO method and only two for real-time PCR) and the contamination level of 0.3 cfu/25 g (two positive samples for the ISO method and one for real-time PCR). Table 14 summarizes the minimal levels of detection estimated after the spiking assay.

4. Discussion

Conventional microbiological detection assays for the major pathogens are available and support the seafood industry (Blackstone et al., 2003). Although these validated methods have proven their efficacy and specificity, they cannot escape the burden of the growth time of the bacterial cultures. In certain circumstances (e.g. in the case of V. vulnificus), they may lead to false negative results (Cañigral, Moreno, Alonso, González, & Ferrús, 2010). Alternative molecular detection methods have thus been developed over the last decade to offer faster methods. In this work, we report the development and optimization of several real-time PCR and multiplex real-time PCR methods. These real-time PCR methods are based on fluorescent labeled probes to further increase the speed of the method as they do not require post-PCR treatments. The target genes were chosen to represent a

| Table 9 |

| Campylobacter jejuni – Results of the spiking assay for the detection of Campylobacter jejuni in seafood samples. |

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Contamination level in 25 g sample (cfu/25 g)</th>
<th>Measured with spiral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Detected†</td>
<td>0</td>
</tr>
</tbody>
</table>

The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

† Estimated value based on enumeration at higher level of contamination.

---

| Table 10 |

| Escherichia coli O157:H7 – Results of the spiking assay for the detection of Escherichia coli O157:H7 in seafood samples. |

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Contamination level in 25 g sample (cfu/25 g)</th>
<th>Measured with spiral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Detected†</td>
<td>0</td>
</tr>
</tbody>
</table>

The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

† Estimated value based on enumeration at higher level of contamination.
Table 11
Salmonella spp. — Results of the spiking assay for the detection of Salmonella spp. in seafood samples.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Contamination level in 25 g sample (cfu/25 g)</th>
<th>Measured with spiral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deduced†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

† Estimated value based on enumeration at higher level of contamination.

The O157 antigen has been extensively used for E. coli O157:H7 detection with specificity for the O157 serogroup among various E. coli strains. It is important to establish the pathogenicity of enterohemorrhagic E. coli isolates, by seeking the genes responsible for virulence expression. For this purpose, protocols have been established by (China, Pirson, & Mainill, 1996) and have to be used on the positive samples obtained after rfe screening.

The detection of Salmonella is based on a specific region of the trr gene. This gene is located in the pathogenicity island 2 and encodes for tetrathionate reductase implicated in tetrathionate respiration (Hensel, Hinsley, Nikolaus, Sawers, & Berkis, 1999). Almost 100% (97%) of the Salmonella strains tested were detected by the PCR proposed here, while no false positive results were observed. This test is based on a previous work performed by Malorny et al. (2004).

The detection of V. parahaemolyticus and V. vulnificus was respectively based on the amplification of tlh and vvp genes. These target genes encode respectively for a thermolabile hemolysin of V. parahaemolyticus and a tissue-damaging metalloprotease of V. vulnificus (Nordstrom, Vickery, Blackstone, Murray, & DePaola, 2007; Wang et al., 2008). In the case of V. parahaemolyticus, it may also be very useful to look for virulence detection genes (tdh/ trh genes) among the isolates. Besides what is described in our study, several protocols have been reported previously (Lo et al., 2008; Nordstrom et al., 2007).

The real-time PCR for V. vulnificus allowed for positive specific detection of all the corresponding strains tested, while no aspecific signal was observed for other species belonging to the Vibrio genus or to other genera. On the other hand, the amplification of the tlh gene led to some significant responses from Vibrio strains belonging to species other than V. parahaemolyticus (V. cholerae, V. alginitolyticus, and V. campbellii). This can be explained by the presence of homologs of the tlh gene in these species. However, the specific response from level as few as one V. parahaemolyticus/ml never gave a Ct higher than 20. This is much lower than the Ct observed for any of the other Vibrio species (Table 5). This is why a Ct threshold value of 20 was selected for this test.

Table 12
Vibrio parahaemolyticus — Results of the spiking assay for the detection of Vibrio parahaemolyticus in seafood samples.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Method</th>
<th>Contamination level in 25 g sample (cfu/25 g)</th>
<th>Measured with spiral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deduced†</td>
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</tr>
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<td></td>
<td></td>
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<td>Cooked shrimps</td>
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<tr>
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<td>Real-time PCR</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Raw shrimps</td>
<td>ISO 21872-1</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Real-time PCR</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Raw mussels</td>
<td>ISO 21872-1</td>
<td>0/4</td>
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</tr>
<tr>
<td></td>
<td>Real-time PCR</td>
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<td>0/4</td>
</tr>
</tbody>
</table>

The contamination level is determined by plating dilutions of the contamination broth before enrichment on appropriate medium and counting cfu after incubation (medium and incubation time as mentioned in the corresponding ISO method).

† Estimated value based on enumeration at higher level of contamination.
This applies to the virulence factors of a pathogenic bacterium found in seafood. The presence of both targets was critical for the virulence of this bacterium (Blackstone et al., 2003; Nordstrom et al., 2007). The presence of both targets was assessed in the same multiplex real-time PCR to establish in one step which virulence pattern the targeted strain belongs to. In total, nineteen strains harboring zero, one, or two target genes were investigated. In each case, the real-time PCR gave a positive signal in C. jejuni strains.

In parallel with these tests, one real-time PCR protocol has been designed to gather information on the putative presence of critical virulence factors of one pathogenic bacterium found in seafood. This applies to the virulence of *V. parahaemolyticus*. The specific targets for *V. parahaemolyticus* are *tdh* and *trh*, coding respectively for two thermostable direct and thermostable-related hemolysins, both critical for the virulence of this bacterium (Blackstone et al., 2003; Nordstrom et al., 2007). The presence of both targets was assessed in the same multiplex real-time PCR to establish in one step which virulence pattern the targeted strain belongs to. In total, nineteen *V. parahaemolyticus* strains harboring zero, one, or two target genes were investigated. In each case, the real-time PCR gave the correct pattern of detection (Table 7), confirming the good specificity and sensitivity of the test.

The last part of this study concerned the strict comparison between the minimal detection level and the sensitivity of the molecular assays and the validated ISO methods. We achieved this by inoculating several seafood matrices with decreasing numbers of each of the studied pathogens. The initial level of contamination was assessed after plating and enumerating the different inoculation doses. The inoculated samples were then subjected to the ISO protocol for microbiological analysis and to the molecular detection with the real-time PCRs on the same 24 h broth enrichment as those stated in the ISO norms. This would enable the concomitant following of the ISO method to confirm the positive real-time PCR results. This is also of paramount importance for isolating the pathogen to conduct epidemiological surveys or for genetic characterization.

The main conclusions of our work were as follows. First, the real-time PCR protocols developed for the detection of six major foodborne pathogens potentially present in seafood lead to a good level of sensitivity and specificity. Second, the results of spiking assays in naturally contaminated seafood products showed that the levels of detection were between 1 and 47 cfu/25 g for the ISO norms and between 1 and 315 cfu/25 g for the real-time PCR protocols. Real-time PCR was more sensitive than the ISO methods in the case of *V. parahaemolyticus*. This difference was also apparent but not so pronounced in the cases of *C. coli* and *C. jejuni*. In contrast, the ISO methods were more appropriate in terms of levels of detection for *Salmonella* in raw shrimps and for *V. vulnificus*, whichever shrimp matrix was seeded.

One recommendation for maximizing the sensitivity of real-time PCR protocols is to use, for the primary steps, the same enrichment broth as those stated in the ISO norms. This would enable the concomitant following of the ISO method to confirm the positive real-time PCR results. This is also of paramount importance for isolating the pathogen to conduct epidemiological surveys or for genetic characterization.

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