International Journal of Food Microbiology 142 (2010) 360–364

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Detection and differentiation of *Vibrio* spp. in seafood and fish samples with cultural and molecular methods

U. Messelhäusser *, J. Colditz, D. Thärigen, W. Kleih, C. Höller, U. Busch

Bavarian Health and Food Safety Authority, Veterinärstr. 2, D-85764 Oberschleißheim, Germany

ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 13 July 2010
Accepted 14 July 2010

Keywords:
Real-time-PCR
Vibrio spp.
Prawns
Shellfish
Fish

ABSTRACT

Vibrio spp. as natural inhabitants of sea- and brackwater of both tropical and temperate regions of the world are commonly found in different kinds of seafood. Even among the three main human pathogenic species *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* most of the isolates from seafood do not carry the different virulence factors responsible for foodborne infections. Therefore, the risk assessment of *Vibrio* spp. in seafood is currently based mainly on the knowledge of the genetic setting of foodborne strains. For the detection and differentiation of *Vibrio* spp. (*V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*) three probe-based multiplex real-time PCR systems were developed and validated. One real-time PCR system simultaneously detects *V. parahaemolyticus, V. cholerae* and *V. vulnificus* on genus level combined with an Internal Amplification Control. The detection limit for the system was between 1 cfu/mL and 10 cfu/mL in pure culture and in different artificially contaminated sample material, e. g. prawns or Alaska Pollock. The other two PCR systems were implemented for the detection of different virulence genes of *V. parahaemolyticus* and *V. cholerae* isolates. The molecular detection systems were applied for the investigation of 338 raw and cooked seafood and fish samples for the presence of the different *Vibrio* spp. The collected data indicate that the PCR systems can be useful for rapid detection and differentiation of *Vibrio* spp. in different food matrices as basis for a preventive consumer protection policy.

1. Introduction

Vibrio spp. are gram-negative and as halophilic bacteria widely spread in sea- and brackwater worldwide. Only a few species, especially *V. parahaemolyticus*, are regularly linked to human foodborne infections caused by consumption of raw, undercooked or recontaminated seafood, but there are also occasional reports of foodborne or waterborne infections caused by environmental *Vibrio* or *Vibrio*-like spp., e. g. *V. mimicus* (Shah and Deokule, 2006), *V. algenolyticus* (Gómez-León et al., 2005; Yoder et al., 2008), and *Grimontia (Vibrio) hollisae* (Edouard et al., 2008).

Even among the three main pathogenic species *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* most of the food and environmental isolates do not carry the currently known different virulence factors, so a well-founded risk assessment of the presence of *Vibrio* spp. in seafood requires the knowledge of the genetic setting of foodborne isolates. Most of the *V. parahaemolyticus* strains associated with human disease carry the thermostable direct haemolysin gene (*tdh*), responsible for the Kanagawa phenomenon, and/or the TDH-related haemolysin gene (*trh1* and *trh2*). Clinical strains of *V. cholerae*, associated with epidemic and pandemic human cholera, produce a potent enterotoxin (CTX), encoded by the *ctxAB* locus (Maiti et al., 2006) in combination with the central regulatory gene *toxR,* which increases *ctx* gene expression (Miller and Mekalanos, 1984).

Due to the possibility that *Vibrio* spp. with currently unknown or atypically virulence factors may cause foodborne infections we developed a two-step screening system for the detection of the three main pathogenic *Vibrio* spp. The first step contains a cultural method in combination with a probe-based multiplex real-time PCR system for the detection of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* on species level used directly from the enrichment broth and for confirmation of isolates. This real-time PCR system is based on the detection of the *toxR* gene specific for *V. parahaemolyticus*, the *sodB* gene, specific for *V. cholerae* and the *vvha* gene specific for *V. vulnificus*. Confirmed isolates were checked with two real-time PCR systems for the presence of different virulence genes of *V. parahaemolyticus* and *V. cholerae*. In case of a positive screening result an enumeration method on a chromogenic medium in combination with a molecular confirmation was added.

The detection method was checked for specifity and sensitivity and used for the investigation of 338 raw and cooked seafood and fish samples from different sources in Bavaria.

* Corresponding author. Tel.: +49 89 31560 314; fax: +49 89 31560 110.
E-mail address: ute.messelhaeusser@lgl.bayern.de (U. Messelhäusser).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.07.020

2. Materials and methods

2.1. Bacterial strains

The reference strains *V. parahaemolyticus* ATCC 43996 and *V. vulnificus* ATCC 27562 and a *V. cholerae* strain, isolated and confirmed by sequencing in our laboratory, were used for developing the real-time PCR systems. Thirty bacterial strains and two fungal strains were checked with every real-time PCR system for exclusivity and 21 different *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* strains for inclusivity. The strains are listed in Table 1. Strains were grown either on nutrient or blood agar.

2.2. Cultural methods

Different cultural methods for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, described in literature were tested to get an enrichment medium and conditions for the simultaneous detection of the two halophilic organisms, *V. parahaemolyticus* and *V. vulnificus*, and *V. cholerae* combined with the real-time PCR methods. For the non-selective enrichment step tryptic soy broth (TSB) containing 2% NaCl and alkaline peptone water (APW) according to the standard methods for the detection of *V. parahaemolyticus* and *V. vulnificus* of the U.S. Food and Drug Administration [FDA] (Kaysner and DePaola, 2004) and the Canadian Microbiological Methods Committee (Warburton, 2006), and marine broth (MB) described by Zobell (1941) were tested under various conditions. For isolation of the different *Vibrio* spp. we tested blood agar, thiosulfate-citrate-bile salts-sucrose (TCBS) agar and the CHROMagar Vibrio (CV) (CHROMagar Microbiology, France), a chromogenic medium described by Hara-Kudo et al. (2001) in parallel.

For further studies we applied a 1:10 dilution of the different sample materials in MB with an enrichment temperature of 37 °C for 24 h. In a second step, 0.1 mL of the enrichment broth culture was plated on both blood agar and CV agar and was incubated at 37 °C for 24 h.

2.3. Primer and probe design

Primers and probes were designed using the freely available Primer3-software (http://frodo.wi.mit.edu). The sequences of different genes of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* were obtained from GenBank (see also Table 2). The primer and probe sequences for the three multiplex real-time PCR systems are shown in Table 2. Initially, the different primer and probe sequences were tested for specificity with BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and showed a 100% similarity for each primer and probe set only with the specific gene or organism. The real-time PCR conditions were optimized for each primer and probe set separately. These conditions were adapted for the use as a multiplex assay with the Stratagene MX 3000P and MX 3005P (Agilent Technologies, USA).

The multiplex real-time PCR system for the detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* at species level contained a heterologous Internal Amplification Control (IAC) based on the pUC 19 plasmid as described previously (Messelhäusser et al., 2007).

2.4. DNA extraction

From 1 mL of the enrichment medium DNA was extracted and purified using the Maxwell® automated purification system (Promega, Germany). For the investigation of isolates we used a thermal lysis step for 15 min at 99 °C.

2.5. Amplification

The real-time PCR assay for the detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* at species level in combination with the IAC was run in a 25 µL volume using the following reaction mix components (final concentrations): 2 × Brilliant Multiplex MasterMix (12.5 µL per reaction; Agilent Technologies, USA), DNAse free H_2O, 250 nm for each of the VP_toxR, VC_sodB and VV_vvha forward and reverse primer, 100 nm for each of the VP_toxR, VC_sodB and VV_vvha probes, 300 nm for each of the IAC_pUC 19 forward and reverse primer, 100 nm for the IAC_pUC 19 probe, 1 fg of the pUC 19 plasmid (Fermentas, Germany) for the IAC and 5 µL DNA template.

The two other multiplex real-time PCR systems for the detection of different toxins genes were also prepared in a 25 µL volume using the following reaction mix components (final concentrations): 2 × Brilliant Multiplex MasterMix (12.5 µL per reaction; Agilent Technologies, USA), DNAse free H_2O, 250 nm for each of the VP_thd, Vp_trh1, VP_trh2, VC_toxR and VC_ctx forward and reverse primer, 200 nm for each of the probes and 5 µL DNA template.

Amplification was done with the following two-step temperature profile: an initial denaturation step for 15 min at 95 °C followed by 45 cycles containing a denaturation step for 10 s at 95 °C and a combined primer annealing and extension step for 60 s at 55 °C.

2.6. Specificity and sensitivity

Each real-time PCR system was checked for specificity with the bacterial strains listed in Table 1. The sensitivity of the assays (as simplex and as multiplex assay) was tested using pure culture and artificially contaminated samples.

2.7. Standard curves

Standard curves for every simplex real-time PCR system and the multiplex systems (Fig. 1) were generated to check the efficiency, precision and sensitivity of the different systems. The standard curves were generated with a six-fold DNA serial dilution starting with 1 ng of purified DNA. The reaction efficiency was calculated by using the

Table 1
Specificity tests of the PCR assays using target and non target strains.

Strains* tested	Number of strains	*V. parahaemolyticus*, *cholerae* and *vulnificus* at genus level			*V. parahaemolyticus* virulence genes			*V. cholerae* virulence genes	
		vvha	*toxR*	*sodB*	*tdh*	*trh1*	*trh2*	*ctx*	*toxR*
Fungi strains	2	0	0	0	0	0	0	0	0
Gram-positive bacteria	10	0	0	0	0	0	0	0	0
Gram-negative bacteria other than *Vibrio* spp.	15	0	0	0	0	0	0	0	0
Other Vibrio spp.	5	0	0	0	0	0	0	0	0
V. parahaemolyticus	11	0	11	0	2	1	2	0	0
V. cholerae	5	0	0	5	0	0	0	2	5
V. vulnificus	5	5	0	0	0	0	0	0	0

* Strains from the German resource center for biological material (DSMZ), Bra unschweig, Germany, from the Institute for fish and fishery products, Cuxhaven, Germany, from the Institute of Hygiene and environment, Hamburg, Germany and from the Bavarian Health and Food Safety Authority, Oberschleißheim, Germany.