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Development of a real-time PCR assay with an internal amplification control for detection of Gram-negative histamine-producing bacteria in fish

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ABSTRACT

Prompt detection of bacteria that contribute to scombrotoxin (histamine) fish poisoning can aid in the detection of potentially toxic fish products and prevent the occurrence of illness. We report development of the first real-time PCR method for rapid detection of Gram-negative histamine-producing bacteria (HPB) in fish. The real-time PCR assay was 100% inclusive for detecting high-histamine producing isolates and did not detect any of the low- or non-histamine producing isolates. The efficiency of the assay with/without internal amplification control ranged from 96–104% and in the presence of background flora and inhibitory matrices was 92/100% and 73–96%, respectively. This assay was used to detect HPB from naturally contaminated yellowfin tuna, bluefish, and false albacore samples. *Photobacterium damselae* (8), *Plesiomonas shigelloides* (2), *Shewanella sp.* (1), and *Morganella morganii* (1) were subsequently isolated from the real-time PCR positive fish samples. These results indicate that the real-time PCR assay developed in this study is a rapid and sensitive method for detecting high-HPB. The assay may be adapted for quantification of HPB, either directly or with an MPN-PCR method.

1. Introduction

Scombrotoxin (or histamine) fish poisoning is one of the leading causes of food-borne illness associated with consumption of fish world-wide (Dewaal et al., 2006). The illness is caused by the consumption of fish containing high levels of histamine and/or other biogenic amines (e.g. putrescine, cadaverine). Histamine is formed in fish by certain spoilage microorganisms capable of producing the enzyme histidine decarboxylase. The histidine decarboxylases produced by these bacteria catalyze the conversion of free histidine, naturally present in the fish muscle, to histamine. Scombroid fish, which belong to the families of *Scomberesocidae* and *Scombridae* (e.g. tuna, mackerel), have most often been implicated in histamine fish poisoning but other non-scombroid fish (e.g. mahi-mahi, bluefish) have also been implicated (Taylor, 1986). The US Food and Drug Administration (FDA) considers fish containing ≥50 ppm histamine to be decomposed and fish with levels of 500 ppm or greater to be a human health hazard (FDA, 2001; Lehane and Olley, 2000). The incubation period of histamine fish poisoning is usually short, ranging from several minutes to several hours after ingestion and symptoms are usually mild and self limiting, subsiding within a few hours. Most common symptoms include diarrhea, flushing and sweating, nausea and headache while some less common symptoms may include stomach pain, oral burning sensation, vomiting, a feverish sensation, dizziness, tight chest, respiratory distress, and facial swelling (Taylor, 1986).

There are two distinct classes of histidine decarboxylases that exist in bacteria; those found in Gram-positive bacteria which require a pyruvoyl prosthetic group and those found in Gram-negative bacteria which require a pyridoxal phosphate cofactor (Recsei and Snell, 1970; Snell and Recsei, 1981; Tanase et al., 1985). Gram-positive histamine-producing bacteria are primarily found in fermented food products such as cheese, wine, and beer (Landete et al., 2008). Gram-negative enteric and marine bacteria are the main contributes to histamine-formation in fish products. These bacteria have been classified based on the ability to produce histamine under ideal culture conditions (Kim et al., 2003). *Morganella morganii*, *Enterobacter aeroegenes*, *Raoultella planticola*, and *Photobacterium damselae* are all high histamine-producing species that produce over 1000 ppm in culture broth. *Hafnia alvei* and *Citrobacter freundii* are low-histamine producing species that generate less than 500 ppm under the same conditions (Allen et al., 2005; Bjornsdottir et al., 2009; Tsai et al., 2004).

Histamine has been implicated as the main contributor to scombrotoxin fish poisoning and quantitative/qualitative test kits and chromatographic methods have been developed for its detection (Cinquina et al., 2004; Frattini and Lionetti, 1998; Hwang et al., 1997; Rogers and Staruszkiweicz, 2000). In order to develop

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effective mitigation strategies, it is necessary to understand the relationship between histamine and histamine-producing bacteria. Microbiological differential media based on pH indicators have been developed for detection and quantification of histamine-producing bacteria with various results (Mavromatis and Quantick, 2002; Niven et al., 1981; Yamani and Untermann, 1985; Yoshinaga and Frank, 1982). Methods based upon these media are time consuming and have been reported to have high false-positive and false-negative responses (Baranowski et al., 1985; Bjornsdottir et al., 2009; Chen et al., 1989). More recently, PCR-based methods have been developed for the detection of Gram-positive and Gram-negative histamine-producing bacteria. Multiple primers sets have been reported for amplification of the histidine decarboxylase gene from Gram-negative bacteria (Kanki et al., 2002; Rivas et al., 2005, 2006; Takahashi et al., 2003). Real-time PCR has the ability to quantify DNA template concentration and is considerably less time consuming than traditional PCR. An internal control nucleic acid molecule can be incorporated into the assay to prevent reporting of false negative results. A real-time PCR assays exist for Gram-positive histamine-producing bacteria (Fernandez et al., 2006; Ladero et al., 2008; Nannelli et al., 2008) but such a method has not yet been developed for Gram-negative histamine-producing bacteria.

We describe here the development of the first real-time PCR method with an internal amplification control targeting the histidine decarboxylase gene of Gram-negative histamine-producing bacteria. This assay is intended for detection of histamine-producing bacteria from fresh and decomposed fish. The specificity of the assay was tested against 148 histamine and non-histamine producing bacteria. In addition to pure culture testing, this assay was also applied to the detection of histamine-producing bacteria (HPB) in naturally contaminated fish samples.

2. Materials and methods

2.1. Bacterial strains

A total of 148 histamine (79) and non-histamine (69) producing Gram-negative bacteria were used to develop and examine the specificity of the primers and probe (Table 1). These strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and from scombrotoxin-forming fish [yellowfin tuna (*Thunnus albacares*), mahi-mahi (*Coryphaena hippurus*), bluefish (*Pomatomus saltatrix*), and wahoo (*Acanthocybium solandri*)] as previously described by Bjornsdottir et al. (2010, 2009).

2.2. Culture conditions and template preparation

For pure culture work, isolates were inoculated into 5 ml of tryptic soy broth (BD; Franklin Lakes, NJ) with the addition of 2% NaCl (Fisher Scientific, Pittsburg, PA; $TSBN_2$) and incubated at 35 °C for 18–24 h. For generation of standard curves by real-time PCR, pure cultures were serially diluted in saline (0.85% NaCl), 0.1 ml was spread plated on tryptic soy agar (BD) containing 2% added NaCl ($TSAN_2$), and inoculated plates were incubated at 35 °C for 24 h. For mixed cultures, a 0.25 ml aliquot from each overnight culture of *Morganella morganii*, *Raoultella planticola*, *Enterobacter aerogenes*, and *Photobacterium damselae* was mixed together and serially diluted in saline (0.85% NaCl), 0.1 ml was spread plated on $TSAN_2$ and plates were incubated at 35 °C for 24 h. Cell suspensions from each of the saline dilutions were heated to 100 °C for 10 min, centrifuged in a micro-centrifuge (Centrifuge 5424, Eppendorf, Hauppauge, NY) at 13,000 × *g* for 2 min and the supernatant was used in the real-time PCR assay. For specificity testing, 1 ml of overnight culture was centrifuged at 13,000 × *g* for 2 min, resuspended in 1 ml of sterile nuclease free water (Ambion/Applied Biosystems, Carlsbad, CA) and boiled as described above. To confirm histamine production by each strain, single colonies were inoculated into tryptic soy broth containing 2% NaCl, 2% histidine hydrochloride monohydrate (MP Biomedical, Solon, OH) and 0.00005% pyridoxal phosphate (Sigma, St. Louis, MO; TSB+) or marine broth (BD; Franklin Lakes, NJ) with 2% histidine. For optimization of the real-time PCR assay, DNA was isolated from cultures of each strain using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) in accordance with manufacturer instructions. The extracted DNA concentration and purity was determined by measuring the absorbance at 260 and 280 nm (NanoDrop; Wilmington, DE, USA). The DNA samples were diluted in sterile nuclease free water (Ambion/Applied Biosystems, Carlsbad, CA) to a stock concentration of 5 ng/ul.

Table 1
Real-time PCR primers and probes sequences used in assay.

Primer or Probe	Sequence (5′ to 3′)	Modification[a]
hdc Forward	TCH ATY ARY AAC TGY GGT GAC TG	None
hdc Reverse	CCR TTR GTN ACR TAV CCC CA	None
hdc Probe	AAC TCN TTY GAY TTY GAR AAR GAR G	5′FAM to 3′ MGBNFQ
IAC Forward	ATGGGTGCCGTTCGAGC	None
IAC Reverse	GAGACGATGCAGCCATTCG	None
IAC Probe	TCTCATGCGTCTCCCTGGTGAATGTG	5′Cy5 to 3′BHQ2

[a] FAM, 6-carboxyfluorescein; MGBNFQ, minor groove binding nonfluorescent quencher; BHQ2, black hole quencher 2.

2.3. Primers and fluorogenic probe design

GenBank was searched for sequences of the gene encoding for the pyridoxal-phosphate histidine deacarboxylase in Gram-negative bacteria. Sequences were aligned using Bioedit Sequence Alignment Editor (Hall, 1999) and a consensus sequence was generated. Two conserved regions were identified and primers designed in these regions using FastPCR software (Helsinki, Finland) corresponding to position 249–271 and 369–388 of the *hdc* gene from *Morganella morganii* (GenBank accession #J02577). The size of the amplified PCR product was anticipated to be 139 bp. The fluorogenic probe was adapted from primer 106 originally designed by De las Rivas et al. (2005) corresponding to position 297–321 of the *hdc* gene. A minor groove binding (MGB) protein was added to the 3′ end to increase the melting temperature of the probe. Primer and probe sequences are listed in Table 1.

2.4. Real-time PCR amplification

Real-time PCR was optimized for protocol parameters, fluorescence detection parameters and reaction components for the detection of Gram-negative histamine-producing bacteria in 25 μl reaction volumes using the following reaction mixture: 1 × PCR amplification buffer (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$ (Roche, Indianapolis, IN), 200 nM of each of the deoxynucleoside triphosphates (Roche, Indianapolis, IN), 500 nM each of the *hdc* forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 300 nM of the probe (Applied Biosystems, Foster City, CA), 1.25 U platinum Taq DNA polymerase (Invitrogen), 2 μl of internal amplification control (IAC) DNA (DePaola et al., 2010; Vickery et al., 2006), nuclease free H_2O, and 0.1–10 ng or 2 ul DNA template.

Real-time PCR thermal cycling was conducted using a Cepheid SmartCycler II system (Sunnyvale, CA). The optimum cycling parameters were: 95 °C hold for 120s for initial denaturation of the