Development of molecular-based methods for determination of high histamine producing bacteria in fish

Kristin Björnsdóttir-Butler a,1, Gregory E. Bolton a, Lee-Ann Jaykus b, Patricia D. McClellan-Greenc, David P. Green a,b,c,d

a Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Center for Marine Sciences and Technology, 303 College Circle, Morehead City, North Carolina 28557, United States
b Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, 100 Schauf Hall, Raleigh, North Carolina 27695, United States
c Department of Environmental and Molecular Toxicology, North Carolina State University, Center for Marine Sciences and Technology, 303 College Circle, Morehead City, North Carolina 28557, United States

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A B S T R A C T
Histamine (or scombroid) fish poisoning is a significant cause of food borne disease in the United States. In this study, we describe the development of a molecular-based technique which uses digoxigenin (DIG) labeled DNA probes for the detection of Gram negative bacteria producing high amounts of histamine (>1000 ppm). A cocktail of PCR amplification fragments corresponding to a 709 bp fragment of the histidine decarboxylase (hdc) gene of four high producing bacteria (Morganella morganii, Enterobacter aerogenes, Raoultella planticola and Photobacterium damselae) was DIG-labeled and screened against a strain bank of 152 Gram negative bacteria isolated from scombroid fish and their harvest environment. The probe cocktail reacted specifically (100%) with the high histamine producing strains but failed to react with low histamine producers and non-producers. To further evaluate the feasibility of the approach, fish homogenate inoculated with known concentrations of four high histamine producing bacterial strains was plated on modified Niven’s medium (culture method) and trypticase soy agar supplemented with 2% NaCl (for colony lift hybridization). The colony lift hybridization counts did not differ significantly from the level of the initial inoculum (p=0.05), while the modified Niven’s counts were significantly lower (p<0.05) than either inoculum or colony lift counts. The use of digoxigenin (DIG) labeled DNA probes with colony lift hybridization shows promise for accurate and specific enumeration of histamine producing bacteria in scombroid fish.

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1. Introduction

Scombroid poisoning is caused by the consumption of fish containing hazardous levels of histamine and other biogenic amines (putrescine and cadaverine). Allergic-like symptoms such as headache, dizziness, swelling of the tongue, nausea, vomiting, diarrhea, and stomach pain occur rapidly after ingestion of contaminated product (Jansen et al., 2003; Maintz and Novak, 2007). The symptoms are usually short-lived and recovery is complete.

Histamine is produced by certain spoilage microorganisms through action of the enzyme histidine decarboxylase (hdc) which converts the amino acid histidine to histamine. There are two different classes of hdc enzymes. The first group is found in eukaryotic cells and Gram negative bacteria, while the second is found in Gram positive bacteria; both have different coenzymes associated with them (Boeker and Snell, 1972; Recsei and Snell, 1984). Histamine is produced by a wide range of bacteria but the major histamine producing bacteria in fish are Gram negative mesophilic enteric and marine bacteria (Kim et al., 2003c). Examples include strains of Morganella morganii, Enterobacter aerogenes, Raoultella planticola, Raoultella ornithinolytica and Photobacterium damselae, some of which are particularly high histamine producers, yielding ≥1000 ppm histamine in broth when cultured under optimal conditions. Strains of other species, including Hafnia alvei, Citrobacter freundii, Vibrio alginolyticus and Escherichia coli, are weak histamine producers (or non-producers), yielding concentrations <500 ppm under similar culture conditions (Matsushita, 1971; Kim et al., 2001, 2004; Takahashi et al., 2003).

Rapid chilling and refrigeration aimed at preventing microbial growth and subsequent histamine formation is the primary control measure in seafood. For example, the U.S. Food and Drug Administration’s (FDA) seafood HACCP rule includes time and temperature control guidelines, as well as a defect action level (DAL) of 50 ppm

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hi:stamine (FDA, 2001c, although much higher histamine levels (>500–1000 ppm) are generally needed to elicit disease in most humans (Lehane and Olley, 2000; ten Brink et al., 1990). These conditions are exacerbated by the fact that a minority of histamine producing bacteria is capable of growth and histamine production at temperatures as low as 2.5 °C (Emborg et al., 2006; Emborg et al., 2005; Haaland et al., 1990).

Therefore, there is a need for reliable, rapid, and accurate methods for histamine determination and for detection of histamine producing bacteria in scombroid fish (FDA, 2001b). Histamine determination in fish muscle using high pressure liquid chromatography (HPLC) might be considered a “gold standard,” but these methods are complicated, time-consuming, and expensive (Shakila et al., 2001). Alternatively, the commercial industry uses immunoassay test kits to distinguish seafood products that contain less than 50 ppm histamine from those that contain more than 50 ppm; these methods are, however, not quantitative.

For detection of histamine producing bacteria, the Niven’s media is the most common method employed (Niven et al., 1981). This differential growth medium contains the pH indicator bromocresol purple as well as tryptone, L-histidine hydrochloride and a few other components. Histamine accumulation, which occurs during the growth of the target organism(s), results in an elevated pH and a media color change which allows discrimination of colonies representing bacteria which are presumptively positive for histamine formation.

Although Niven’s media is a good presumptive tool for screening bacterial colonies, it has been reported to produce a number of false positive isolates which must be subsequently confirmed by histamine determination (Baranowski et al., 1985; Chen et al., 1989; Lopez-Sabater et al., 1996b). In addition, low pH of the media (pH 5.3) is also thought to inhibit growth of some histamine producing bacteria (Actis et al., 1999; Yoshinaga and Frank, 1982), resulting in false negative isolates that later test positive for histamine production. Some researchers have modified pH, incubation temperature and time to improve the performance of Niven’s medium, particularly for the quantification of histamine producing bacteria amongst a high background of harmless microflora (Chen et al., 1989; Mavromatis and Quantick, 2002).

More recently, molecular methods have been developed for the detection of Gram negative histamine producing bacteria in fish. For example, Takahashi et al. (2003) and Kanki et al. (2002) developed a nucleic acid amplification method targeting 709 bp and 724 bp fragments of the histidine decarboxylase (hdc) gene. Another PCR method was developed for the detection of the prolific histamine producer M. morganii, based on the amplification of the variable region of the 16S rDNA region (Kim et al., 2003a; Kim et al., 2003b). Similar methods have been developed for amplification of the hdc gene of Gram positive histamine producing bacteria (Alves et al., 2002; Le Jeune et al., 1995). Methods such as these can be used to confirm bacterial cultures which screen positive on Niven’s media, but they do not eliminate the need to verify histamine production by the bacteria strains.

Colony lift hybridization is a method which uses the combined power of microbiological culture with nucleic acid hybridization. This method is uniquely suited to situations in which the performance of selective and/or differential media is less than perfect, as it provides more accurate quantitative results because the target organism can be confirmed without the need for sub-culturing. Colony lift hybridization methods have been applied for the detection and quantification of various food borne pathogens including Vibrio parahaemolyticus, V. vulnificus, Campylobacter spp., and Listeria monocytogenes (Carroll et al., 2000; DePaola et al., 2003; Rice et al., 1996). In a recent report for V. parahaemolyticus, investigators claimed that the colony lift approach was faster and more efficient than the most probable number (MPN) methods frequently used for detection and quantification of this pathogen (FDA, 2001a; Gooch et al., 2001; Nishibuchi et al., 1985).

Our hypothesis is that molecular-based probes can be used in conjunction with colony lift hybridization methods to quantify Gram negative histamine producing bacteria in scombroid fish by virtue of the specificity of a probe targeting the hdc gene of these organisms. Therefore, the purpose of this study was to develop molecular-based probes for the detection of high histamine producing bacteria in fish. In initial phases, labeled DNA probes were evaluated using dot blot hybridization for their ability to detect (specificity) 152 seafood-derived Gram negative bacteria demonstrating varying degrees of histamine production. The assay was then converted to a colony lift hybridization format, the efficiency of which was assessed using artificially inoculated fish samples and compared to the modified Niven’s agar method.

2. Materials and methods

2.1. Bacterial cultures

The culture library of 152 Gram negative histamine and non-histamine producing bacteria described in Björnsdóttir et al. (2009) was used in this study. Histamine production for each bacterial strain was evaluated using the HPLC method (Cinquina et al., 2004). The detection limit for this method was 125 ppm based on a standard curve produced using a histamine suspension serially diluted in the range of 2.5–200 ppm. The primary histamine producing bacterial strains used in this study are shown in Table 1.

2.2. Culture conditions

Media and media components were obtained from Becton Dickinson (BD; Franklin Lakes, NJ) unless otherwise specified. For pure culture work, strains were cultured in trypticase soy broth (TSB) or agar (TSA) containing 2% NaCl (EMD, Gibbstown, NJ; TSA(A)N2). For confirmation of histamine production, bacteria were cultured in TSB containing 2% histidine (L-histidine hydrochloride monohydrate; Acros, Morris Plains, NJ); 2% NaCl and 0.0005% pyridoxal-HCl pH 6.5 (Alexis, Plymouth Meeting, PA; TSB-). In both instances, incubation was done at 37 °C for 24–48 h. Inoculum to be used in the fish seeding studies was prepared by combining equal volumes of overnight cultures of M. morganii, R. planticola and E. aerogenes and P. damselae, washing by centrifugation at 13,700×g and resuspending the pellet in 1 ml of saline (0.85% NaCl). For quantification of histamine producing bacteria in inoculated fish homogenates, samples were serially diluted in saline and spread plated on modified Niven’s agar in accordance with the formulation of Mavromatis and Quantick (2002) (0.5% tryptone, 0.5% yeast extract, 2.7% histidine, 0.5% NaCl, 0.1% CaCO3 (Mallinckrodt, Hazelwood, MO), 3% agar, and 0.006% bromocresol purple (Fisher Scientific, Pittsburgh, PA) pH 5.3); incubation was done at 30 °C for 24 h. This protocol was used to facilitate detection of high histamine producing isolates. The same samples and corresponding dilutions were also spread plated on TSA and with incubation at 30 °C for 24 h followed by colony lift hybridization as described below.

2.3. DNA extraction

DNA from pure cultures (for dot blot hybridization) was isolated using the UltraClean™ Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA) in accordance with manufacturer instructions. Briefly, overnight bacterial cultures were concentrated by centrifugation and then chemically/mechanically lysed in bead solution containing detergents. The DNA was then bound to a silica spin filter, washed and recovered in Tris buffer. Absorbance was recorded at 260 and 280 nm (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) in a 96-
well quartz plate and DNA concentration and purity were calculated using standard formulae.

2.4. Histamine decarboxylase (hdc) gene sequencing

A 709 bp fragment of the histidine decarboxylase (hdc) gene from 17 high producing strains [e.g., M. morgani (ATCC 35200, ATCC 25830, BR119, BO249, BO255, HPP301, HPP309); R. planticola (ATCC 43176); R. ornithinolytica (HPP15, HPP19); E. aerogenes (ATCC 13048, HPP14); and P. damselae (BR100, BR107, BR132, BR147, BR165)] was amplified for subsequent sequencing using primers described by Takahashi et al. (2003) [hdc- (5'-TCH ATY AAR TGY GGT GAC TGG RG-3') and hdc-r (5'-CCC ACA KTA TBA RWG GDG TRT GRC C-3')] [Integrated DNA Technologies, Coralville, IA]. Amplification was performed in 50 µl reactions that included 25 µl PCR master mix (50 units/ml Taq DNA polymerase, 400 µM of each of the four deoxynucleoside triphosphate, reaction buffers, 3 mM MgCl2, pH 8.5 (all provided by Promega Corp., Madison, WI)], 75 pmol of each primer, and 20 ng DNA template. A total of 40 amplification cycles (94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min) was done using a GTC-2 thermal cycler (Precision Scientific, Chicago, IL). PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining under UV transillumination. Product size was confirmed using a 100 bp molecular weight marker (Invitrogen, Carlsbad, CA).

The 17 amplification products were cloned using the pCR2.1 TA cloning kit using One Shot Competent Escherichia coli TOP10 cells (Invitrogen, Carlsbad, CA) in accordance with manufacturer instructions. From each cloned library, 10 white colonies were picked randomly. The clones were screened by PCR for the presence of hdc inserts and by restriction mapping using EcoRI (Invitrogen, Carlsbad, CA). For each strain, one clone confirmed to contain the hdc fragment was selected for sequencing. Plasmids were purified using the PureLink HiQ Mini Plasmid Purification kit (Invitrogen, Carlsbad, CA) and sequenced by Davis Sequencing, Inc. (Davis, CA).

2.5. Probe development and labeling

Six-candidate degenerate probe sequences were designed by alignment of the sequences corresponding to the 17 partial hdc clones described above using the FastPCR program (University of Helsinki, Helsinki, Finland). The degenerate probes were purchased from Integrated DNA Technologies (Coralville, IA) and end labeled using the DIG Oligonucleotide 3’-End Labeling Kit (Roche, Indianapolis, IN) in accordance with manufacturer instructions.

In addition, two additional probe fragments (709 bp and 249 bp) were produced by PCR DIG-labeling of the DNA template obtained from four high histamine producing strains: M. morgani (ATCC 35200), P. damselae (mahi-mahi BR100, E. aerogenes (ATCC 13048) and R. planticola (ATCC 43176). To make these probes, a 709 bp DNA sequence corresponding to positions 249–958 of the hdc gene of M. morgani (GenBank accession number J02577) was labeled using primers previously described by Takahashi et al. (2003) [hdc- (5'-TCH ATY AAR TGY GGT GAC TGG RG-3') and hdc-r (5'-CCC ACA KTA TBA RWG GDG TRT GRC C-3')] by sequence alignment of the partial hdc gene from M. morgani, was DIG-labeled by PCR using the primers hdc-r (Takahashi et al., 2003) and a primer designed specifically for this study (designated hdc2- (5’-AYG CBG AYG CSG CDC TRA GYG GHA TGA -3’)) by sequence alignment of the partial hdc gene from M. morgani, P. damselae, E. aerogenes, and Raoultella spp. (GenBank accession numbers FJ69557–73). Amplifications for the DIG-PCR labeling were carried out as described above.

2.6. DNA dot blot

To determine the specificity of the DNA probes, dot blot hybridization was done using DNA derived from pure cultures of all 152 histamine and non-histamine producing isolates used in this study. Dot blots were prepared using a 96-well Bio-Dot microfiltration
apparatus (Biorad, Hercules, CA) and positively charged nylon membranes (Roche, Indianapolis, IN). Membranes were pre-washed in sterile distilled water before dot blot. Aliquots (2 µl) of purified DNA at a standardized concentration of 10 ng/µl were denatured by the addition of 0.4 M NaOH/10 mM EDTA and heated at 100 °C for 10 min followed by immediate icing. The denatured DNA sample was applied to the positively charged nylon membrane by vacuum filtration followed by washing with 0.4 M NaOH, rinsing in 2× SSC, air drying and UV cross-linking (UVP, Upland, CA).

2.7. Colony lift

Twenty g tissue samples excised from the dorsal loins of mahi-mahi (Coryphaena hippurus) and yellowfin tuna (Thunnus albacares) were homogenized in 180 ml of saline (0.85% NaCl) and inoculated with serially-diluted overnight cultures of histamine producing bacteria so as to obtain concentrations ranging from 2 to 5 log10 CFU/g. Fish samples both before and after inoculation were spread plated to modified Niven’s agar (for quantification of histamine producing bacteria by culture) and TSAN2 (for quantification using colony lift hybridization). Colony lifts were done using positively charged nylon membranes (Roche, Indianapolis, IN) which were applied to agar plates for 1 min, after which the membranes were exposed sequentially to denaturation solution (0.5 M NaOH/1.5 NaCl) for 5 min, neutralization solution (1.5 M NaCl/1.0 M Tris-HCl, pH 7.4) for 5 min, and 2× SSC for 5 min. The membranes were then air dried and UV cross-linked. Prior to pre-hybridization, each membrane was incubated at 37 °C for 1 h in 0.5 ml proteinase K (Roche, Indianapolis, IN) diluted to a concentration of 2 mg/ml in 2× SSC. In all experiments, a mixed strain suspension of M. morganii, R. planticola, E. aerogenes and P. damselae was used as a positive control while mixed cultures of Escherichia coli and Shewanella putrefaciens were used as negative controls.

2.8. Hybridization

Dot blot and colony lift membranes were pre-hybridized in DIG Easy Hybridization reagents (Roche, Indianapolis, IN) at 40 °C for 1 h. The pre-hybridization solution was replaced with the hybridization solution supplemented with 5.0 µl/ml DIG-labeled PCR probe mix (1.25 µl/ml each probe) and the membranes were incubated overnight at 40 °C. The membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 5 min and twice in 0.5× SSC/0.1% SDS at 55 °C for 15 min. After a brief final wash in a proprietary washing buffer, the membranes were blocked using a solution of 100 mM maleic acid/150 mM NaCl (pH 7.5) containing 1× blocking reagent for 30 min at room temperature and then exposed to the same buffer containing anti-digoxigenin–alkaline phosphatase Fab fragments (1:5000) for 30 min. After washing twice in washing buffer for 15 min, membranes were developed by exposure to 20 µl/ml of NBT/BCIP suspended in detection buffer (0.1 M Tris–HCl/0.1 M NaCl, pH 9.5).

2.9. Statistical analysis

Dot blot experiments were performed in duplicate and colony lift evaluation was done in triplicate. The STATISTICA (StatSoft, Inc., Tulsa, OK) software package was used for all statistical analyses. Analysis of variance (ANOVA) with discrimination of means using the Tukey honest significant difference (HSD) test was used to compare counts obtained by the Niven’s method and colony lift hybridization within each inoculation level. The effect of the sample matrix (tuna and mahi-mahi homogenates) on colony lift hybridization as applied to inoculated fish homogenates was analyzed using the general linear model (GLM) approach. The analysis was considered statistically significant if p ≤0.05.

3. Results

3.1. Partial hdc nucleotide sequences from high histamine producing strains

Segments of the hdc genes from 17 high histamine producing bacteria were cloned, sequenced and submitted to GenBank under the following accession numbers: M. morganii strain ATCC 35200, FJ469557; M. morganii strain ATCC 25830, FJ469558; M. morganii strain BR119, FJ469559; M. morganii strain BO249, FJ469560; M. morganii strain BO255, FJ469561; M. morganii strain HPP301, FJ469562; M. morganii strain HPP309, FJ469563; R. planticola strain ATCC 43176, FJ469564; R. ornithinolytica strain HPP15, FJ469565; R. ornithinolytica strain HPP19, FJ469566; E. aerogenes strain ATCC 13048, FJ469567; E. aerogenes strain HPP14, FJ469568; P. damselae strain BR100, FJ469569; P. damselae strain BR107, FJ469570; P. damselae strain BR132, FJ469571; P. damselae strain BR147, FJ469572; P. damselae strain BR165, FJ469573. The hdc gene from the seven M. morganii strains exhibited sequence similarity in the range of 96–99%. The three Raoultella spp. and the two E. aerogenes hdc gene sequences exhibited between 87 and 99% and 98% sequence similarity, respectively. Finally, the five P. damselae hdc genes exhibited 99% sequence similarity. When comparing the nucleic acid sequence identities between the four high histamine producing species (M. morganii, R. planticola, E. aerogenes and P. damselae), similarities ranged from 73 to 78%.

3.2. Evaluation of probe performance

Six-candidate 3’ end-labeled degenerate probes were examined for their ability to detect these strains by dot blot hybridization. Three degenerate probes showed a high degree of non-specific binding which limited their usefulness. The other three degenerate probes showed weakly positive signals for eight high histamine producing strains within the species of M. morganii, E. aerogenes, R. ornithinolytica and P. damselae (data not shown). Unfortunately, efforts to improve signal intensity by increasing DNA concentration, probe concentration, or probe labeling efficiency were unsuccessful and these probes were not evaluated further.

Because the degenerate probes performed poorly, two sets of PCR-labeled probes (one 709 bp in size and the other 249 bp) were generated separately using the DNA derived from the high histamine producers M. morganii, R. planticola, E. aerogenes and P. damselae. When these probes were screened individually by dot blot hybridization against each of the 152 isolates in the strain bank, none of them were able to detect all of the histamine producing isolates. However, when the 709 bp probes were mixed in equal proportion, probe concentration, or probe labeling efficiency was unsuccessful and these probes were not evaluated further. Because the degenerate probes performed poorly, two sets of PCR-labeled probes (one 709 bp in size and the other 249 bp) were generated separately using the DNA derived from the high histamine producers M. morganii, R. planticola, E. aerogenes and P. damselae. When these probes were screened individually by dot blot hybridization against each of the 152 isolates in the strain bank, none of them were able to detect all of the histamine producing isolates. However, when the 709 bp probes were mixed in equal proportion, their cocktail was able to detect all 73 of the high histamine producing strains (≥1000 ppm) and none of the low (126–500 ppm) or none of the low (126–500 ppm) or 73 non- (<125 ppm) histamine producing bacteria (Fig. 1; Table 1). Similar results were observed when the 249 bp probes were mixed in equal proportion, but this probe mix showed a higher degree of non-specific binding than did the 709 bp hdc-probe mix (Table 1). Therefore the 709 bp hdc-probe mix was used in subsequent colony lift hybridization experiments.

3.3. Enumeration of histamine producing bacteria in inoculated fish samples by colony lift hybridization

To examine a practical application of the 709 bp hdc-probe mix for the quantification of histamine producing bacteria in fish samples, tissue homogenates (tuna and mahi-mahi) were inoculated with known numbers (in the range of 2.0 to 5.0 log CFU/g) of a cocktail of four high histamine producing strains (M. morganii, R. planticola, E. aerogenes, and P. damselae). These samples were analyzed using both modified Niven’s media and colony lift hybridization using the 709 bp hdc-probe mix. The background flora in uninoculated mahi-mahi and tuna samples were similar and ranged from 4.1 to 4.5 log CFU/g. There
was no statistically significant difference \((p > 0.05)\) when comparing the levels of the initial inocula and counts of histamine producing bacteria using colony lift hybridization, irrespective of the level of inoculum or fish species (Fig. 2A–B). There were, however, statistically significant differences \((p < 0.05)\) when comparing the quantification of histamine producing bacteria by modified Niven’s media to that obtained by colony lift hybridization, again irrespective of inoculum level or fish type. The 709 bp hdc-probe mix did not appear to react with any of the indigenous microbial population in the seafood homogenate, with the absence of a non-specific binding when the assay was applied to uninoculated control samples (Fig. 3).

### 4. Discussion

Molecular-based detection methods for histamine producing bacteria are almost exclusively based on detection of the \(hdg\) gene and/or the genes of other amino acid decarboxylases, usually by PCR (Kim et al., 2003a,b; Rivas et al., 2005, 2006; Takahashi et al., 2003). At the time of this study’s initiation, a PCR-based approach to quantify Gram negative histamine producing bacteria of concern in scrombroid fish had not been reported. This study provides proof-of-concept that molecular-based methods can be developed for the quantification of histamine producing bacteria in fish tissue, and these newer methods may overcome some consistent problems with culture-based methodology. Indeed, the detection of histamine producing bacteria by traditional culture-based methods is both time consuming and relatively non-specific. For instance, previous studies have noted that colonies identified as histamine producers using Niven’s differential media are actually false positive from 15 to 63% of the time, particularly when using extended incubation periods (Fletcher et al., 1998; Lopez-Sabater et al., 1996b). In addition, Niven’s agar formulations can substantially underestimate the levels of histamine producing bacteria, presumably due to the low pH of the medium (pH 5.3), which may exclude growth of some histamine producers (Niven et al., 1981).

The probe mix created by PCR amplification and labeling of the \(hdg\) gene of four high histamine producing strains (\(M.\) morganii, \(R.\) planticola, \(E.\) aerogenes, and \(P.\) damselae) performed extremely well when applied to strains producing >1000 ppm histamine under ideal conditions. Specifically, high histamine producing strains of \(M.\) morganii, Providencia rustigianii, Proteus mirabilis, Raoultella planticola, \(R.\) ornithinolytica, Enterobacter aerogenes, \(E.\) gergoviae and \(P.\) damselae showed 100% specificity using the probe mix. Likewise, the probe mix detected none of the non-histamine producers in our culture collection. However, two \(H.\) alvei strains and four \(C.\) freundii strains producing low levels of histamine (126–
500 ppm) were not detected using this probe mix. Others have observed similar problems detecting low producing strains using molecular-based methods. For example, Takahashi et al. (2003) were unable to detect a *Citrobacter braakii* strain by amplification of the *hdc* gene despite the fact that the strain produced 311 ppm histamine in broth. Overall, the *hdc*-probe mix used in our study was able to discriminate between high producers and non-producers, but all low histamine producing strains would be designated as non-producers using this probe mix.

Nonetheless, the existence of bacteria producing low levels of histamine has been equivocally reported (Lopez-Sabater et al., 1996a; Ozogul and Ozogul, 2007). Therefore, failure to detect such strains using culture or molecular-based assays may have several explanations. Firstly, the low levels of histamine produced by these strains may occur as a result of non-specific activity of other amino acid decarboxylases. In fact, arginine decarboxylases from *Salmonella typhimurium* and *Escherichia coli* have been shown to metabolize histidine in addition to their natural substrates (Blethen et al., 1968; Cundell et al., 1991). Therefore, low levels of histamine may be produced even in the absence of the *hdc* gene. Secondly, the *hdc* gene may be plasmid-associated in low histamine producing bacteria, as has been observed for *Vibrio anguillarum* (Di Lorenzo et al., 2003; Landete et al., 2008). To date, investigators have not sequenced the *hdc* gene from a low producing bacterial strain so we have limited information upon which to base our current speculations.

In our study, we observed substantially lower counts using modified Niven’s media compared to the TSAN2 media in conjunction with colony lift hybridization, which produced counts that were not significantly different from expected levels of histamine producing bacteria based on inoculum titer. This may be a function of the neutral pH of the TSAN2 used to culture the bacteria before colony lift as compared to the modified Niven’s media, whose reduced pH (5.3) might have inhibited the growth of some of the histamine producing strains. Mavromatis and Quentick (2002) recommended incubation of Niven’s agar plates at 30 °C for 48 h (rather than 37 °C for 24 h) to allow for the growth of low histamine producers, even though high producers showed pronounced color change within 24 h at 37 °C. We modified our culture conditions by incubating the Niven’s agar plates at 30 °C for 24 h to favor the recovery of the high histamine producers used as our inoculums. The lower pH in combination with reduced temperature and incubation time may help explain the lower counts observed using modified Niven’s media as compared to colony lift hybridization. In addition, delayed color development may be an alternative explanation (Chen et al., 1989).

The Niven’s method does, however, have its advantages. It is certainly a low cost technique requiring minimal equipment and skill level of the analyst. Colony lift hybridization requires more expensive equipment and a higher skill level, but may allow the analyst to bypass additional steps necessary to confirm presumptively positive colonies obtained using Niven’s media formulations. Both methods excel in detecting those bacteria which are able to produce toxic levels of histamine (>500 ppm), while the detection of strains producing low levels of histamine remains problematic. Even though the U.S. FDA guidelines specify 50 ppm histamine as the defect action level, epidemiological data suggests that histamine quantities exceeding 500 ppm are required to cause scombrototoxic fish poisoning (Lehane and Olley, 2000; ten Brink et al., 1990). Therefore, the importance of the low histamine producers with respect to disease causation is currently unknown and needs further investigation.

In conclusion, we report proof-of-concept evidence that colony lift hybridization can be used to detect and quantify high histamine producing bacteria in scombroid fish tissue. The method was not able to detect or discriminate between low- and non-histamine producing bacteria. Future efforts should address the need for detection of low
histamine producers and evaluation of molecular methods to detect histamine in naturally-contaminated scombroid fish and environmental samples. It is our hope that the use of molecular-based probes used in conjunction with colony lift hybridization will be a useful tool in future studies addressing risk reduction and a routine method for screening of fish samples for excessive levels of histamine producing bacteria.

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