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ORIGINAL ARTICLE

Development of molecular approach based on PCR assay for detection of histamine producing bacteria

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Abstract Histamine fish poisoning becomes highly concern not only in public health but also economic aspect. Histamine is produced from histidine in fish muscles by bacterial decarboxylase enzyme. Several techniques have been developed to determine the level of histamine in fish and their products but the effective method for detecting histamine producing bacteria is still required. This study was attempted to detect histamine producing bacteria by newly developed PCR condition. Histamine producing bacteria were isolated from scombroid fish and determined the ability to produce histamine of isolated bacteria by biochemical and TLC assays. PCR method was developed to target the histidine decarboxylase gene (*hdc*). The result showed that fifteen histamine producing bacterial isolates and three standard strains produced an amplicon at the expected size of 571 bp after amplified by PCR using Hdc_2F/2R primers. Fifteen isolates of histamine producing bacteria were classified as *M. morganii*, *E. aerogenes*, and *A. baumannii*. The lowest detection levels of *M. morganii* and *E. aerogenes* were 10^2 and 10^5 Cfu/mL in culture media and 10^3 and 10^6 Cfu/mL in fish homogenates, respectively. The limit of detection by this method was clearly shown to be sensitive because the primers could detect the presence of *M. morganii* and *E. aerogenes* before the histamine level reached the regulation level at 50 ppm. Therefore, this PCR method exhibited the potential efficiency for detecting the *hdc* gene from histamine producing bacteria and could be used to prevent the proliferation of histamine producing bacteria in fish and fish products.

Keywords Histamine producing bacteria · PCR detection · Scombrotoxin · *Morganella morganii* · *Enterobacter aerogenes*

Introduction

Marine fish are valued as a healthy diet because they provide beneficial nutrition including essential amino acids, vitamins, and minerals (Lund 2013; Puwastien et al. 1999). However, a large portion of seafood intoxications usually correlates with fish and its products (Huss 1997; Lipp and Rose 1997). Scombrotoxin or histamine fish poisoning is usually implicated in the illness associated with consumption of mishandle fish or fish products (Lehane and Olley 2000; Santos 1996; Tao et al. 2011). The symptoms of histamine poisoning generally resemble with the symptoms from food allergies (Taylor et al. 1989) which are nausea, vomiting, diarrhea, oral burning sensation, hives, itching, rash, and hypotension (Lehane and Olley 2000; Taylor et al. 1989). According to the US FDA regulation, the contamination level of histamine in fish and fish products must be lower than 5 mg of histamine per 100 g of fish (50 ppm) (Lehane and Olley 2000). Scombroid fish, the member of Scomberesocidae and Scombridae family (tuna, mackerel, bonito, and jack) and some non-scombroid fish including mahi-mahi, sardines, pilchards, anchovies, herring, marlin, and blue fin are commonly implicated in the outbreaks of histamine fish poisoning because their muscle

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containing high level of free amino acid histidine which is preserved as a substrate for bacterial histidine decarboxylase enzyme (Hungerford 2010; McLauchlin et al. 2005; Taylor et al. 1989). The majority of histamine producing bacteria which have been isolated from implicated fish are Gram-negative bacteria such as *Morganella morganii*, *Hafnia alvei*, *Proteus vulgaris*, *Proteus milabilis*, *Enterobacter aerogenes*, *Raoultella planticola*, *Raoultella ornithinolytica*, *Citrobacter freundii*, *Pseudomonas fluorescens*, and *Photobacterium damselae* (Chen et al. 2008; Björnsdóttir-Butler et al. 2010). These bacteria are capable of converting the free amino acid histidine in fish muscle into histamine by the activity of their histidine decarboxylase enzyme (Björnsdóttir-Butler et al. 2010; Taylor et al. 1989). Normally, the contamination of histamine producing bacteria is observed after catching (Takahashi et al. 2003). The prolific histamine producers such as *M. morganii*, *E. aerogenes*, *R. planticola*, and *P. damselae* can generate hazardous level of histamine in a short period when stored under inappropriate temperature. However, the spoilage sign may not be detected even though the amount of histamine reaches the unsafe level (Björnsdóttir-Butler et al. 2011; Taylor 1986). Currently, several standard analytical methods for observing the amount of histamine in fish and fish products such as high performance liquid chromatography (HPLC), liquid chromatography with mass spectrometric (LC-MS), and enzyme-linked immunosorbent assay (ELISA) are created (Hungerford 2010). However, all these methods can detect the amount of histamine after the growth of histamine producing bacteria higher than 10^6 CFU/mL (Hungerford 2010; Kung et al. 2012). Although the differential medium developed by Niven and co-worker (1981) is a simple method to screen for histamine producing bacteria, the high rates of false positive and false negative are still observed (Ababouch et al. 1991; Björnsdóttir-Butler et al. 2010). The molecular approach based on polymerase chain reaction (PCR) is becoming an interesting method to detect foodborne pathogens including histamine producing bacteria (Björnsdóttir-Butler et al. 2011; Kung et al. 2012; Takahashi et al. 2003). Previous studies have demonstrated PCR-based method to detect Gram-negative histamine producing bacteria. (Björnsdóttir et al. 2009; Björnsdóttir-Butler et al. 2010; De las Rivas et al. 2005; Takahashi et al. 2003). Interestingly, the sensitivity of PCR detection methods based on the *hdc* specific primers have not been reported on *M. morganii* which is one of the predominant strains of histamine producing bacteria isolated from implicated fish. The real time PCR method has been employed to detect histamine producing bacteria in fish and fish products; however, this method is still expensive and required specialists to perform the complicated analytical procedures (Björnsdóttir-Butler et al. 2011; Ferrario et al. 2012). For this reason, the practical and reliable methods for detecting histamine producing bacteria are required in order to monitor and control the growth of histamine producing bacteria.

In this study, histamine producing bacteria were screened from tuna and mackerel purchased from the convenient stores in Thailand. The specific primers to histidine decarboxylase gene (*hdc*) were designed according to the conserved region from various strains of histamine producing bacteria. The sensitivity of the primers was determined both in culture broth and fish homogenate conditions. This study was the first report on the sensitivity of PCR detection assay for detecting *M. morganii* in culture medium and fish homogenate by using the *hdc* specific primers. Moreover, the correlation between the growth of histamine producing bacteria and histamine formation was also evaluated and compared with the detection limit of PCR assay.

Materials and methods

Microorganisms and isolation of histamine producing bacteria

Three reference strains of histamine producing bacteria (*Morganella morganii* ATCC 25830, *Enterobacter aerogenes* ATCC 13048, and *Acinetobacter baumannii* ATCC 19606) and five strains of non-histamine producing bacteria (*Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *Enterica* serovar Typhimurium ATCC 13311, *Salmonella enterica* subsp. *Enterica* serovar Choleraesuis ATCC 10708, *Salmonella enterica* subsp. *Enterica* serovar Typhi, and *Shigella sonnei*) were obtained from the Department of Medical Science, Ministry of Public Health, Thailand and the Department of Microbiology, Faculty of Pharmacy, Mahidol University, Thailand. All cultures were grown in tryptic soy agar (TSA; Difco Laboratories, Dickinson and Company, USA) at 37 °C for 18 h. Fish samples were purchased from retail markets and convenient stores in Bangkok, Thailand. The selected samples were short bodies mackerel (*Rastrelliger brachysoma*), indian mackerel (*Rastrelliger kanagurta*), pacific mackerel (*Scomber japonicas*), king mackerel (*Scomberomorus cavalla*), and longtail tuna (*Thunnus tonggol*). In order to isolate the histamine producing bacteria, 10 g of each sample including skin and muscles were aseptically removed and grinded after that were placed into 90 mL of 0.1 % peptone water. The mixture was homogenized by shaking at 250 rpm for 60 min at 25 °C. A homogeneous sample was transferred into 9 mL of 0.1 % peptone water and ten-fold serial dilution was performed. Histamine producing bacteria were initially screened by pour plate method using Niven's medium (Niven et al. 1981). The plates were incubated at 25 °C for 48 h and purple or purple halo colonies were selected for further studies.

Confirmation of histamine production by thin layer chromatographic assay

The histamine producing ability of selected bacteria was determined by thin layer chromatographic assay (TLC) according to the method of Valls et al. 2002 with some modifications. The selected colonies were transferred into TSB (Tryptic soy broth) supplemented with 1 % L-histidine and incubated at 25 °C for 24 h without shaking. After designated time, each sample was spotted on the TLC plate (Silica gel $60F_{254}$; Merck, Germany). Then, the plate was developed in the mobile phase containing the mixture of chloroform, methanol, and ammonia in the ratio of 25:15:4. The developed plate was sprayed with Pauly's reagent by mixing solution A (9.55 g of sulfanilic acid in 2.5 N hydrochloric acid) with solution B (5 % aqueous sodium nitrite) at ratio 1:1 and then the plate was air-dried. After the plate was completely dried, solution C (10 % aqueous sodium carbonate) was sprayed over the plate in order to display histamine spot. The color of spot appeared in dark orange.

Amplification of histidine decarboxylase gene (*hdc* gene) by PCR method

Hdc_2F (5′-TGG-GGT-TAT-GTS-ACC-AAT-GG-3′) and Hdc_2R (5′-GTR-TGG-CCG-TTA-CGY-GAR-CC-3′), the specific primers for amplifying the *hdc* gene, were constructed according to the conserved region of *hdc* gene from 11 strains of histamine producing bacteria which were deposited in GenBank consisting of *Klebsiella planticola* (M62746), *M. morganii* (AB256290 and J02577), *Proteus vulgaris* (AB083204), *Pseudomonas fluorescens* (CP003041 and Y09356), *Pseudomonas entomophila* (CT573326), *E. aerogenes* (M62745), *Photobacterium damselae* (AB084251, AB259289) and *Photobacterium phosphoreum* (AB259287). The size of amplification product was 571 bp. The PCR reaction was performed in the reaction mixtures; 20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM Mg_2SO_4, 0.1 % TritonX-100, 20 pmol of each primer, 0.2 mM each of four dNTPs, 0.5 U of *Taq* DNA polymerase (NEB, England) and 10 ng of DNA template. The amplification thermal cycles were carried out for 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. The initial denaturation and final extension temperatures were 94 °C for 4 min and 72 °C for 4 min, respectively. The PCR products were visualized by 1.3 % agarose gel electrophoresis at 80 V for 45 min in 1 × TAE.

Determination of the specificity of Hdc_2F/R primers

The genomic DNA of histamine producing and non-histamine producing strains was prepared by the Puregene® Core Kit B; QIAGEN. Briefly, overnight culture was centrifuged at 14, 000 rpm for 5 s to pellet cells, then resuspended in cell lysis solution and incubated at 80 °C for 5 min. The RNase A solution was added (incubated at 37 °C for 15 min) after that protein precipitation solution was added and centrifuged at 14, 000 rpm for 3 min. The supernatant was mixed with isopropanol and centrifuged at 14,000 rpm for 1 min. The DNA pellet was washed with 70 % ethanol and the DNA pellets were resuspended in DNA hydration solution. For the specificity test, genomic DNA was used as a template in order to amplify a *hdc* gene according to the PCR condition as previously described. Moreover, the nucleotide sequences of an amplicon were analyzed and compared to the nucleotide sequences of *hdc* gene in GenBank.

Evaluation of the sensitivity of Hdc_2F/R primers

The prolific histamine producing bacteria, *M. morganii* ATCC 25830 and *E. aerogenes* ATCC 13048 were used to evaluate the sensitivity of the primers. The bacteria were cultured in TSB with shaking at 150 rpm for 18 h at 25 °C. After that overnight culture was transferred into TSB and incubated until the turbidity of tested bacteria reached the exponential growth phase at OD_{600} 1.0. The viable cell count of the tested bacteria was performed, then the bacteria were diluted with 0.1 % peptone water to obtain the concentration in the range of 10 to 10^8 Cfu/mL. In this study, DNA templates for PCR assay were obtained from whole cells, cell lysates, and genomic DNA. Moreover, the sensitivity of Hdc_2F/R primers was determined in an artificial condition by inoculation of fish muscle. The inner muscle was aseptically transferred into TSB and an aliquot of inoculum was inoculated into fish homogenates. The final concentration of the bacteria in fish homogenates was adjusted to 10 to 10^8 Cfu/mL. The sensitivity of the primers was determined against three different types of DNA templates as described above.

PCR detection of histamine producing bacteria

In order to detect histamine producing bacteria in culture medium by PCR technique, the concentration of starting inocula was diluted to 10^2 Cfu/mL and incubated at 25 °C for 16 h. The growth rate and histamine production of *M. morganii* and *E. aerogenes* were determined at 0, 2, 4, 6, 8, 10, 12, 14, and 16 h. The viable cell counts were evaluated by spread plate methods on TSA. The supernatant at each time point was spotted on TLC plate and the spot of histamine was visualized by sprayed with Pauly's reagent. The amount of histamine was quantified by computerized scanning densitometer (CARMAX) under white light at 520 nm. The standard curve of histamine was created with the concentration in the range of 25 to 250 μg/mL. For detecting the *hdc* gene of *M. morganii* and *E. aerogenes* with Hdc_2F/2R primers, culture medium

was collected at each time point and DNA template was extracted by boiling method.

Identification of genus and species of histamine producing bacteria by 16S rRNA sequencing and phylogenetic tree analysis

The 16S rRNA gene was amplified by using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR reaction mixture was composed of 20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM Mg_2SO_4, 0.1 % TritonX-100, 20 pmol of each primer, 0.25 mM each of four dNTPs, 0.5 U of *Taq* DNA polymerase (NEB, USA), 10 ng of DNA template and made the final volume to 50 μL by sterilized DDW. The thermocycling program was started with initial denaturation at 94 °C for 3 min and followed by 35 thermal cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s. Finally, the final extension was done at 72 °C for 3 min. The PCR products were visualized by 1.0 % agarose gel electrophoresis and the amplicon was purified by using QIAquick® gel extraction kit. The purified PCR products were ligated with pGEM®-T cloning vector and transformed into *E. coli* strain DH5α after that the plasmids were extracted and purified by using QIAprep® Spin Miniprep Kit. The plasmids that contained the inserted DNA were selected to analyze the nucleotide sequence by using universal primers (T7 and SP6). The nucleotide sequences were analyzed by SolGent Co., Ltd., Korea. The results were compared with the 16S rRNA sequences of bacterial type strains which were deposited in EzbioCloud database (www.ezbiocloud.net/eztaxon).

The evolutionary pattern and diversity of histamine producing bacteria were demonstrated by phylogenetic tree based on the distance method. The 16S rRNA sequences were aligned with MUSCLE 3.8.3. The evolutionary history was inferred using the neighbor–joining method (Saitou and Nei 1987). The maximum composition likelihood method was used to calculate the differences in nucleotides (Tamura et al. 2004). Finally, the evolution analysis was conducted in MEGA6 (Tamura et al. 2013).

Results and discussion

Screening of histamine producing bacteria

To select for histamine producing bacteria, Niven's medium was used as the selective and differential medium (Niven-JR et al. 1981). After screening, the total of 500 isolated colonies showed purple or purple halo in color on Niven's medium. Among those, only 15 isolates showed histamine-positive results based on TLC assay which were 5 isolated from indian mackerel (IM1, IM2, IM3, IM4, and IM5), 4 isolates from king mackerel (KM1, KM2, KM3, and KM4), 4 isolates from longtail tuna (LT1, LT2, LT3, and LT4), and 2 isolates from short bodies mackerel (SB1 and SB2) (Table 1). In this study, screening by using differential medium exhibited high level of false positive results because the principle of this medium for separating histamine producing and non-histamine producing bacteria was the change of medium or colony color from yellow to purple in respond to the pH indicator. Therefore, pH shift causing the purple color on medium or bacterial colonies might arise from other alkaline compounds which were not exactly of histamine (Ababouch et al. 1991; Bover-Cid and Holzapfel 1999; Takahashi et al. 2003). TLC method was selected to verify the result from differential medium because it did not require complicated instruments and could be directly observed the presence of histamine with low incidence of false positive (Ababouch et al. 1991).

The specificity analysis of Hdc_2F/R primers

The specificity of the designed primers was tested against three standard strains of histamine producing bacteria and five strains of non-histamine producing bacteria. All histamine producing bacteria displayed the PCR product at an expected size of 571 bp while non-specific PCR products were obtained from the group of non-histamine producing bacteria (Table 1). The consistent results between PCR and TLC assay were obtained as the isolates demonstrated positive results by TLC assay also showed the PCR product at the expected size in PCR method. Similarly, non-histamine producing bacteria gave negative results in both assays. The nucleotide sequences of the PCR amplicon were identified and compared to the nucleotide sequences of *hdc* gene databases from GenBank. The results indicated that *hdc* nucleotide sequences of three standard strains (*M. morganii*, *E. aerogenes*, and *A. baumannii*) were similar to the *hdc* gene of *M. morganii* ATCC 25820 (FJ469558.1), *E. aerogenes* ATCC 13048 (FJ469567.1), and *A. baumannii* LAC-4 (CP007712.1) at 99 % identity. Interestingly, although the contamination of *A. baumannii* in the marine fish product has been found, the specific primers for detecting *hdc* gene of *A. baumannii* have not been reported (Chen et al. 2010).

The sensitivity of Hdc_2F/2R primers

The sensitivity of Hdc_2F/2R primers was further evaluated by using *M. morganii* and *E. aerogenes*. The results showed that the lowest limit of these primers to detect *M. morganii* was 10^2 Cfu/mL for cell lysates and whole cells, while the primers could detect *M. morganii* genomic DNA at 10^4 Cfu/mL. In the artificial inoculation experiment, the levels of *M. morganii* detection of Hdc_2F/2R primers were 10^3, 10^4, and 10^5 Cfu/mL according to the templates which were prepared from cell lysates, whole cells, and genomic DNA, respectively. However, the sensitivity of tested primers was

Table 1 Isolation of histamine producing bacteria from marine fish samples

Microorganism	Niven's medium	PCR (571 bp)	TLC	Source
M. morganii	+	+	+	ATCC (25830)
E. aerogenes	+	+	+	ATCC (13048
A. baumannii	+	+	+	ATCC (19606)
E. coli	−	−	−	ATCC (25922)
S. Choleraesuis	−	−	−	ATCC (10708)
S. Typhimurium	+	−	−	ATCC (13311)
S. Typhi	−	−	−	Laboratory strain[a]
S. sonei	−	−	−	Laboratory strain[a]
IM 1	+	+	+	Indian mackerel
IM 2	+	+	+	Indian mackerel
IM 3	+	+	+	Indian mackerel
IM 4	+	+	+	Indian mackerel
IM 5	+	+	+	Indian mackerel
KM 1	+	+	+	King mackerel
KM 2	+	+	+	King mackerel
KM 3	+	+	+	King mackerel
KM 4	+	+	+	King mackerel
LT 1	+	+	+	Longtail tuna
LT 2	+	+	+	Longtail tuna
LT 3	+	+	+	Longtail tuna
LT 4	+	+	+	Longtail tuna
SB 1	+	+	+	Short bodies mackerel
SB 2	+	+	+	Short bodies mackerel

[a] The strains were obtained from the department of Microbiology, Faculty of Pharmacy, Mahidol University

decreased for *E. aerogenes*. The highest sensitivity of the primers was obtained from cell lysates and whole cells at 10^5 Cfu/mL and the lower sensitivity was gained when used genomic DNA as a template. In fish homogenates, the best sensitivity of the primers was obtained from cell lysates of *E. aerogenes* at 10^6 Cfu/mL (Fig. 1). In addition, Hdc_2F/2R primers could detect the *hdc* gene of *E. aerogenes* at 10^7 Cfu/mL when whole cells and genomic DNA were used as template. The difference in sensitivity test of these two histamine producing bacteria might occur from dissimilar in some positions of the nucleotides in the conserved region of *hdc* gene and the designed primers were composed of degenerated bases which were created by a variety of histamine producing bacterial strains. Moreover, the lower sensitivity detected in an artificial inoculation might occur from contamination of inhibitor substances in fish such as organic compounds, glycogen, fat, and ion (Kung et al. 2012). Molecular detection methods based on conventional PCR technique have been developed for detecting histamine producing bacteria in fish and fish products. Most studies could detect the broad spectrum of Gram-negative histamine producing bacteria, however, these methods did not mention about the sensitivity of the detection systems (Björnsdóttir et al. 2009; Björnsdóttir-Butler et al. 2010; De las Rivas et al. 2005; Takahashi et al. 2003). Only the report from Kung and co-workers (2012) showed the sensitivity of PCR assay to detect Gram-negative bacteria (*E. aerogenes* and *R. ornithinolytica*) by two sets of *hdc* specific primers which were designed according to the procedures of De las Rivas et al. (2005) and Takahashi et al. (2003). The best sensitivity for detecting Gram-negative histamine producing bacteria in culture broth and fish homogenates was obtained from the primer set of Takahashi et al. (2003) with the detection limit for *E. aerogenes* at concentration of 10^5 Cfu/mL for culture broth and 10^6 Cfu/mL for fish homogenates. The sensitivity of the previously designed primers by Takahashi et al. (2003) was comparable with the sensitivity of Hdc_2F/2R primers in this study for observing the presence of *E. aerogenes* in culture medium and fish homogenates. *M. morganii* was one of the predominant strains of histamine producing bacteria which have usually been isolated from implicated fish, but it was surprising that the sensitivity of PCR detection methods based on the *hdc* specific primers have not been reported. This experiment was the first report on the sensitivity of PCR detection assay for detecting *M. morganii* in culture medium and fish homogenate by using the *hdc* specific primers. The specific primers targeted to 16S rRNA of *M. morganii* were previously designed but these primers presented unsatisfying sensitivity with detection level at the concentration in the range of 10^6 to 10^8 Cfu/mL in fish homogenate condition (Kim et al. 2003a). The real time PCR

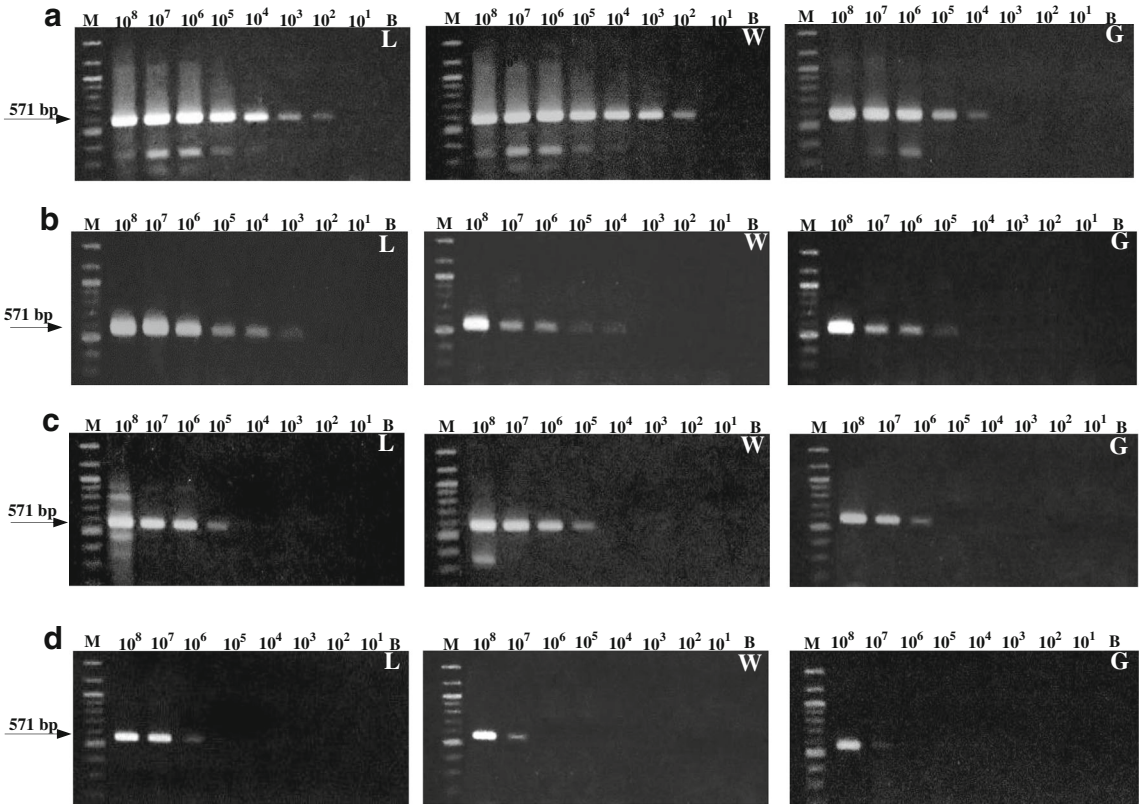

Fig. 1 The sensitivity test of Hdc_2F/2R primers against *hdc* gene of *M.morganii* and *E. aerogenes*. Figure a and b showed the sensitivity of primers against *M. morganii* in culture medium and fish homogenate, respectively. Figure c and d showed the sensitivity of primers against *E. aerogenes* in culture medium and fish homogenate. The abbreviate L, W, and G are represented as the DNA templates which were prepared from cell lysates, whole cells, and genomic DNA, respectively

method was employed to detect the histamine producing bacteria in fish and fish products (Björnsdóttir-Butler et al. 2011; Ferrario et al. 2012). This technique showed the promising efficiency with high sensitivity to detect the presence of high histamine producing strains such as *M. morganii*, *E. aerogenes*, *R. planticola*, and *P. damselae*; however, this method was still expensive and required specialists to perform the complicated analytical procedures.

Correlation of PCR detection limit and histamine production level

The correlation of the growth rate and the ability to produce histamine of histamine producing bacteria was evaluated in the study and the detection limit of Hdc_2F/2R primers was also determined. The histamine amount which was produced by *M. morganii* could be observed at 12 h after the culture reached 6.0×10^8 Cfu/mL with histamine level of 26.71 μg/mL. Additionally, the PCR assay was able to detect the presence of *M. morganii* at concentration of 3.7×10^3 Cfu/mL before the level of histamine content was higher than the regulatory level at 50 μg/mL (Lehane and Olley 2000). In case of *E. aerogenes*, histamine content was rapidly produced during 8–10 h. The histamine level was detectable after 10 h at 109.07 μg/mL when the culture reached 7.46×10^8 Cfu/mL and the histamine content was produced exceeding 250 μg/mL after14 h (Table 2). PCR assay could detect the appearance of *E. aerogenes* at 1.16×10^6 Cfu/mL before the histamine content was detected by TLC densitometer. The results of this study were in conformity with the data from the previous studies from Kung et al. (2012) and Takahashi et al. (2003) which indicated that *M. morganii* and *E. aerogenes* were able to secrete histamine when viable cell counts were higher than 10^7 Cfu/mL in culture medium. Although the artificial inoculation did not perform in this study, the results in sensitivity test of PCR assay indicated that Hdc_2F/2R primers could detect the appearance of *M. morganii* and *E. aerogenes* in fish homogenate when produced histamine at the level that could not be detected by HPLC technique from both histamine producers (Kung et al. 2012; Takahashi et al. 2003).

Identification of genus and species of isolated bacteria by the sequencing of 16S rRNA and phylogenetic tree analysis

The genus and species of isolated histamine producing bacteria in the present study were identified by sequencing the 16S rRNA gene. The 16S rRNA sequences from 15 histamine producers

Table 2 The correlation of the growth rate of histamine producing bacteria and histamine level

Culture	Time (h)	Cfu/mL	Histamine content (μg/mL)	PCR assay
M. morganii	0	80	ND	-
	2	3.70×10^3	ND	+
	4	1.17×10^4	ND	+
	6	7.36×10^5	ND	+
	8	1.45×10^6	ND	+
	10	1.03×10^8	ND	+
	12	6.00×10^8	26.71	+
	14	1.15×10^9	128.81	+
	16	4.73×10^9	187.15	+
E. aerogenes	0	73	ND	-
	2	6.23×10^3	ND	-
	4	4.10×10^4	ND	-
	6	1.16×10^6	ND	+
	8	1.07×10^7	ND	+
	10	7.46×10^8	109.07	+
	12	5.30×10^9	205.87	+
	14	1.46×10^{10}	> 250.00	+
	16	5.86×10^{10}	>250.00	+

were compared to those of well characterized 16S rRNA sequences which were deposited in EzbioCloud database. The results indicated that the 15 isolates of histamine producing bacteria were classified into 3 genera consisting of *Morganella*, *Enterobacter*, and *Acinetobacter* (Table 3). Among of those, 9 isolates were identified as *Morganella morganii* that could be categorized into 2 subspecies, *M. moganii* subsp. *morganii* (IM1, IM3, IM4, IM5, KM2, and LT1) and *M. morganii* subsp. *sibonii* (IM2, KM1, and LT2). The remainders expected for SB2 were classified as *Enterobacter aerogenes* while SB2 was named as *Acinetobacter baumannii*. On the phylogenetic tree that was constructed based on 16S rRNA sequences of 101 bacterial type strains from EzbioCloud database and 15 isolates of histamine producing bacteria were distributed into 14 clusters including out group (Fig. 2). The isolated bacteria were located in the cluster C, F, and L. From the phylogenic tree, the isolated samples in the cluster C, F, and L were closely related with genus *Morganella* spp., *Enterobacter* spp., and *Acinetobacter* spp., respectively. According to the consistent results between the phylogenetic tree and 16S rRNA analysis, *M. morganii* was the most dominant genus of histamine producing bacteria which was isolated from marine fish, followed by *E. aerogenes* and *A. baumannii*. This data was in concordant with previous studies which indicated that *M. morganii* was a main contributor for histamine accumulation in marine fish and it was regularly isolated from incriminated fish or fish products after the outbreak of histamine fish poisoning (Klausen and Huss 1987; Kim et al. 2002; Kim et al. 2003b; Rawles et al. 1996). It was noteworthy that even though *E. aerogenes* was not recognized as the principal histamine producer in histamine fish poisoning outbreaks but it also had an impact on the accumulation of histamine in marine fish and fish products because it was classified as prolific histamine producing bacteria (Kim et al. 2009; Takahashi et al. 2003; Taylor et al. 1978). *A. baumannii* was classified as a weak histamine producer and rarely isolated from incriminated fish when compared with other histamine producing bacteria (Chen et al. 2010).

Conclusions

At present study, the Hdc 2F/2R primers were designed for detecting the *hdc* gene of histamine producing bacteria. The

Table 3 Identification of genus and species of histamine producing bacteria based on 16S rRNA sequence

Isolate	Closest correlative strain	Accession no.	% Identity
IM1	*Morganella morganii* subsp. *morganii*	DQ358131	99.83
IM2	*Morganella morganii* subsp. *sibonii*	DQ358146	98.83
IM3	*Morganella morganii* subsp. *morganii*	DQ358131	99.17
IM4	*Morganella morganii* subsp. *morganii*	DQ358131	99.08
IM5	*Morganella morganii* subsp. *morganii*	DQ358131	99.25
KM1	*Morganella morganii* subsp. *sibonii*	DQ358146	98.83
KM2	*Morganella morganii* subsp. *morganii*	DQ358131	99.50
KM3	*Enterobacter aerogenes*	CP002824	99.75
KM4	*Enterobacter aerogenes*	CP002824	99.75
LT1	*Morganella morganii* subsp. *morganii*	DQ358131	99.00
LT2	*Morganella morganii* subsp. *sibonii*	DQ358146	99.08
LT3	*Enterobacter aerogenes*	CP002824	99.75
LT4	*Enterobacter aerogenes*	CP002824	99.75
SB1	*Enterobacter aerogenes*	CP002824	99.75
SB2	*Acinetobacter baumannii*	ACQB01000091	100.00

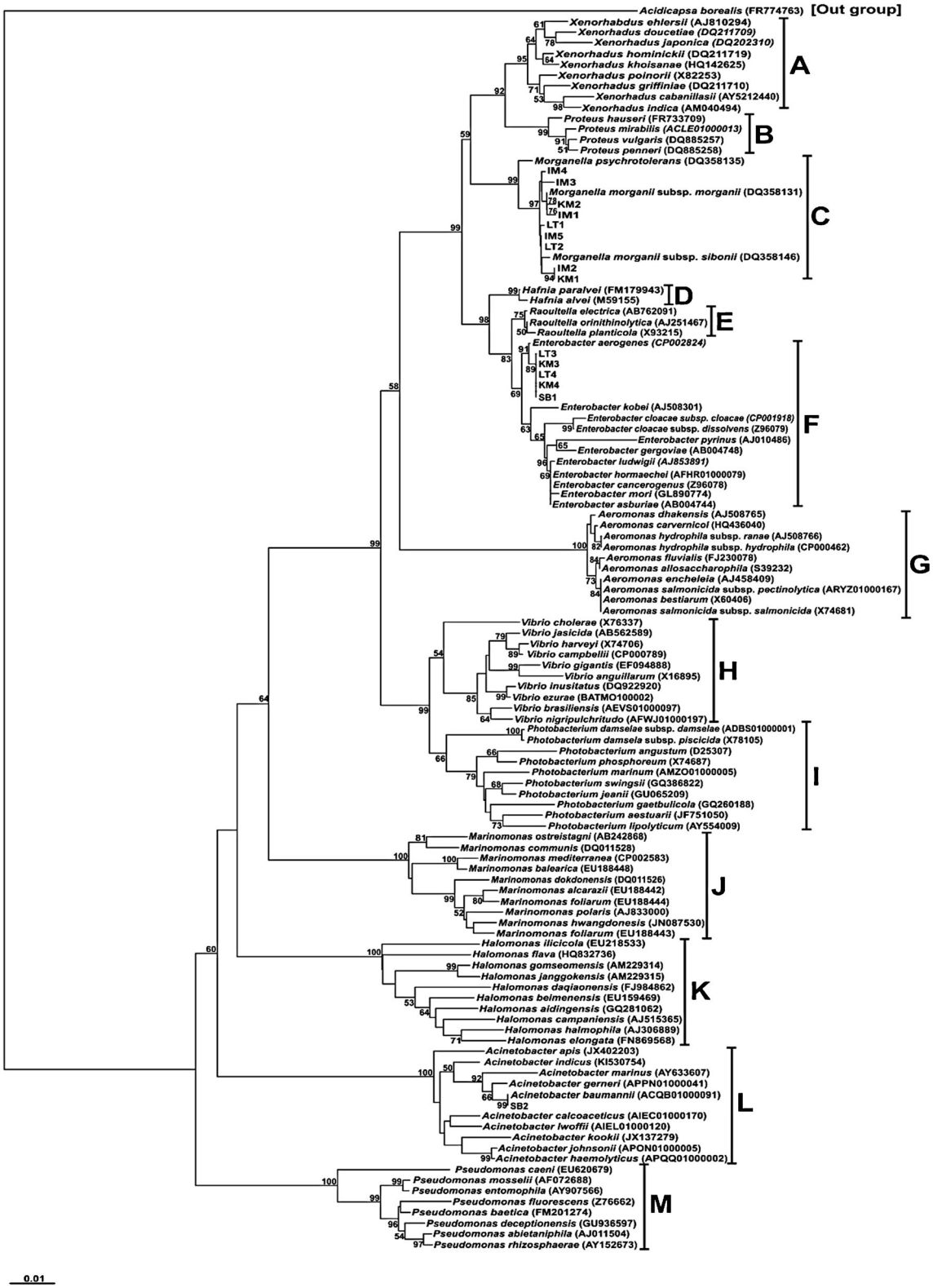

Fig. 2 Evolution patterns of the 15 isolated histamine producing bacteria. The histamine producing bacteria were classified into 3 genera located on different clusters consisting of Cluster A for *Morganella* spp., Cluster F for *Enterobacter* spp., and Cluster L for *Acinetobacter* spp.

primers could detect the *hdc* gene from all tested strains of histamine producing bacteria. According to the specificity and sensitivity test, the designed primers exhibited the outstanding potential for detecting the *hdc* gene from *M. morganii* both in culture and artificial condition. Moreover, Hdc 2F/2R primers were able to detect *M. morganii* and *E. aerogenes* before the

level of histamine reached the control level. For this reason, PCR assay was a suitable method for detecting histamine producing bacteria in order to prevent the hazard from histamine fish poisoning. Further investigation with diversified strains of histamine producing bacteria should be performed.

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