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Polymerase Chain Reaction for the Detection of Histamine-Producing Bacteria Isolated from Taiwanese Foods

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ABSTRACT

It is important to develop rapid methods to detect the presence of histamine producers. In this study, the polymerase chain reaction (PCR) assay yielded a 367-bp DNA amplification fragment from histidine decarboxylase (hdc) of gram-positive bacteria using JV16HC/JV17HC primer, and 534-bp and 709-bp DNA amplification fragments from histidine decarboxylase of gram-negative bacteria using 106/107 and hdc-f/hdc-r primers, respectively. Seven gram-positive histamine-producing strains and 15 gram-negative histamine-producing strains isolated from Taiwanese foods successfully produced the PCR amplification products. The minimum levels of detection of *Enterobacter aerogenes* or *Raoultella ornithinolytica* after the PCR amplification using hdc-f/hdc-r and 106/107 primers were 10^5 and 10^6 CFU/mL in TSB broth, and 10^6 and 10^7 CFU/g in marlin homogenates, respectively. The hdc amplification using hdc-f/hdc-r primer was detected 2 h earlier than the HPLC detection of histamine in TSBH broth or marlin homogenates inoculated *E. aerogenes* or *R. ornithinolytica*. However, the detection of hdc amplification using the 106/107 primer and that of histamine by HPLC occurred at the same sampling time. Therefore, the PCR method could be easily used to detect potential histamine-producing bacteria in foods.

Key words: histamine, PCR, histamine-forming bacteria, histidine decarboxylase gene (hdc)

INTRODUCTION

Scombroid poisoning is a type of food poisoning that results from eating mishandled tuna and related scombroid fish containing elevated levels of histamine[(1)]. Histamine intoxication is probably the best known sanitary problem of food-borne diseases associated with eating fish[(1)]. Scombroid poisoning is usually a mild illness with a variety of symptoms including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin[(1)]. The severity of the symptoms can vary considerably with the amount of histamine ingested and the individual's sensitivity to histamine. Histamine is formed through the proliferation of bacteria that synthesize histidine decarboxylase (hdc) to convert free histidine to histamine[(2)]. While histamine in fermented products, such as wine[(3)], cheese[(4)], douchi[(5)], mustard pickle[(6)] and fish sauce[(7)], is produced by gram-positive lactic acid bacteria and *Staphylococcus* spp., histamine in raw fish products is caused mostly by gram-negative enteric bacteria such as *Morganella morganii*, *Raoultella orithinolytica* and *Enterobacter aerogenes*[(8-10)].

Histamine-producing bacterial species are likely to be introduced into fish and raw materials during handling after capture[(11)] and following temperature abuse. Therefore, it is important to develop rapid methods to detect the presence of histamine producers before dangerous levels of histamine are reached. Niven's medium[(12)] has been most widely used, although high rates of false positives and false negatives are observed, presumably due to the presence of competing bacteria[(13)]. Several improved screening methods have been described[(14,15)]. However, most screening procedures generally involve the use of a differential medium containing a pH indicator. Some reports[(16,17)] described false-positive reactions in some media due to the formation of alkaline compounds or false-negative responses to some bacteria in fermentation. Moreover, conventional cultural assays require 2 to 3 days to complete, making these methods inefficient for the rapid

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detection of histamine-producing bacteria.

Molecular methods for the detection and identification of food-borne pathogens are becoming more widely accepted as an alternative to traditional culture methods. Since histamine is the decarboxylation product of histidine catalyzed specifically by the enzyme histidine decarboxylase, it is possible to develop a molecular detection method that detects the gene responsible for the production of this enzyme. Two different kinds of hdcs were found in gram-negative and gram-positive bacteria. The hdcs of gram-negative bacteria utilize homometric pyridoxal 5'-phosphate (PLP)-dependent enzymes[18], whereas the hdcs of gram-positive bacteria utilize heterometric enzymes that contain an essential pyruvoyl group but no PLP[19]. PCR techniques targeting bacterial histidine decarboxylase genes have been previously designed. Different sets of primers have been developed for detecting gram-positive and gram-negative types. Le Jeune *et al*.[20] developed primer set JV16HC/JV17HC that amplifies a 367-bp fragment for the detection of gram-positive histamine-producing bacteria. Similarly, the two primer sets tested for the amplification of the hdc gene in gram-negative bacteria were hdc-f/hdc-r[21] and 106/107[22], which amplified DNA fragments of 709 and 534 bp, respectively. Rapid detection of gram-negative histamine producers is important for detecting and preventing microbial contamination and high histamine levels during the processing of fish products. However, there was no report in sensitivity associated with the PCR assay using the primer sets hdc-f/hdc-r and 106/107 for the detection of gram-negative histamine producers.

Recently, there were some gram-positive and gram-negative histamine-producing bacteria isolated from Taiwanese foods in our laboratory[5,6,9,23-25]. The objective of this study was to understand whether the previously designed PCR primers and assay were suitable for the amplification of the hdc genes of histamine-producing bacteria isolated from some Taiwanese foods. The sensitivity of the PCR assay was evaluated with the use of cultured broth and marlin homogenate inoculated and serially diluted with gram-negative histamine-producing bacteria. In addition, the correlation was observed between the histamine levels and the hdc gene amplicon of PCR method artificially contaminated cultured broth and marlin homogenate with gram-negative histamine-producing bacteria.

MATERIALS AND METHODS

I. *Bacterial Strains*

The bacterial strains isolated from Taiwanese foods in this study were *Staphylococcus capitis* (five strains) and *S. pasteuri* (two strains) from mustard pickle and douchi products, and *Enterobacter aerogenes* (four strains), *Raoultella ornithinolytica* (nine strains) and *R. planticola* (one strain) from a variety of fish samples, including sailfish fillet, dried milkfish and tuna dumpling. *Lactobacillus* 30a (ATCC 33222), a histamine-producing strain was purchased from the American Type Culture Collection (ATCC). Both *Proteus vulgaris* and *Morganella morganii* strains isolated from albacore tuna were kindly provided by Dr. S. H. Kim[8]. Two strains of *E. aerogenes* (ATCC 13048) and *M. morganii* (ATCC 25830) were obtained from the ATCC. In addition, non-histamine-producing gram-positive strains (four strains) and gram-negative strains (six strains) were isolated from Taiwanese fermented foods and seafood products, respectively [5,6,9,23-25].

II. *Determination of Histamine Production in Bacterial Strains*

The ability of strains to produce histamine was determined by inoculating the isolates in trypticase soy broth (TSB) (Difco) supplemented with 1% L-histidine (TSBH) and incubated without shaking at 35°C for 24 h. One milliliter of the culture broth was taken for the quantitation of histamine. Samples of standard histamine solutions and 1 mL of the culture broth were derivatized with dansyl chloride according to a previously described method[26]. The dansyl derivatives were filtrated through a 0.45-μm filter, and 20-μL aliquots were used for HPLC injection. The contents of histamine in the culture broth samples were determined with a Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) consisting of a Model L-7100 pump, a Rheodyne Model 7125 syringe-loading sample injector, a Model L-4000 UV-Vis detector (set at 254 nm) and a Model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4.6 mm, E. Merck, Darmstadt, Germany) was used for chromatographic separation. The gradient elution program began with acetonitrile : water (50 : 50, v/v) at a flow rate of 1.0 mL/min for 19 min, followed by a linear increase to acetonitrile : water (90 : 10, v/v) at 1.0 mL/min during the next 1.0 min. The acetonitrile-water mix was decreased to 50 : 50 (1.0 mL/min) for 10 min.

III. *DNA Extraction, Primers and PCR Procedures*

With the exception of the *Lactobacillus* 30a strain inoculated in MRS broth (Difco, Detroit, MI, USA) and incubated at 30°C for 24 h, histamine-producing and non-histamine-producing strains were inoculated in TSB broth (Difco) at 30°C for 24 h. The DNA of bacterial culture or fish homogenate was extracted using the Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. To detect the histidine decarboxylase coding gene (hdc) of gram-positive strains, we tested the primer set JV16HC/JV17HC to generate a 367-bp fragment[20] (Table 1). The two primer sets for the amplification of the hdc gene in gram-negative strains were hdc-f/hdc-r[21] and 106/107[22], which amplified DNA fragments of 709 and 534 bp, respectively (Table 1). Amplification was performed in 50-μL reactions that included 25 μL of PCR master mix (50 U/mL Taq DNA polymerase, 400 μM concentrations of each of the four deoxynucleoside triphosphates, reaction buffers,

Table 1. Primers tested for the specific detection of gram-positive and negative histamine-forming bacteria

Target gene[a]	Primer name	Sequence 5´→3´[b]	Amplicon size (bp)
hdc (for Gram＋)	JV16HC	AGATGGTATTGTTTCTTATG	367
	JV17HC	AGACCATACACCATAACCTT	
hdc (for Gram－)	106	AAYTCNTTYGAYTTYGARAARGARG	534
	107	ATNGGNGANCCDATCATYTTRTGNCC	
hdc (for Gram－)	hdc-r	CCCACAKCATBARWGGDGTRTGRCC	709
	hdc-f	TCHATYARYAACTGYGGTGACTGGRG	

[a]hdc: histidine decarboxylase.
[b]Y = C or T; R = A or G; W = A or T; D = G, A or T; N = A, C, G or T.

and 3 mM of $MgCl_2$, pH 8.5) (Promega Corp., Madison, WI, USA), and 75 pmol of each primer. Amplifications were carried out for 35 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 1 min) in a MultiGene Gradient Thermal Cycler. PCR products were separated on a 1.5% agarose gel at 100 V in 0.5 x TBE (89 mM of Tris-borate, 2 mM of EDTA, pH 8.3) for 50 min. The gels were stained with ethidium bromide (0.3 μg/mL) and visualized with a UV transilluminator (UVP, Upland, CA, USA). The product size was confirmed by comparison with 100-bp molecular weight markers (Invitrogen, Carlsbad, CA, USA).

IV. *Primers Specificity Analysis*

The specificity of the previously designed primer JV16HC/JV17HC was tested against 8 gram-positive histamine-producing strains and 4 non-histamine-producing strains (Table 2). Likewise, the specificities of primer sets 106/107 and hdc-r/hdc-f were tested against 19 gram-negative histamine-producing strains and 6 non-histamine-producing strains (Table 2). A 1.0-mL portion of the overnight cell culture for each strain was centrifuged and a 1-μL portion of DNA was used as a PCR template. PCRs were carried out in duplicate as described above to determine the specificity of the assay.

V. *Sensitivity of the PCR Detection for Gram Negative Histamine-Producing Bacteria*

For the sensitivity analysis of bacterial cultures, *E. aerogenes* 05 or *R. ornithinolytica* 06 were cultured overnight at 30°C and serially diluted 10-fold with 0.1% peptone water to obtain concentrations ranging from 10^2 to 10^8 CFU/mL. One-milliliter portions of the diluted cell cultures were used for DNA extraction. A 1-μL portion of DNA template was subjected to PCR amplification using 106/107 and hdc-r/hdc-f primers as described above.

For the sensitivity analysis of fish homogenate, a block of freshly caught marlin fish was purchased from a commercial processor. The block was aseptically processed under a vertical laminar flow hood in our laboratory. The outer surface of the block was cut away to remove any potential cross-contamination from the surface to the inner muscle. A 30-g portion of internal muscle tissue was blended with 120 mL of 0.1% peptone in water in a sterile blender. *E. aerogenes* 05 or *R. ornithinolytica* 06 cultures cultured overnight and serially diluted 10-fold with 0.1% peptone water were inoculated into the fish homogenate. Final inoculation levels were adjusted to 10^3 to 10^9 CFU/g. A 1.0-mL portion of fish homogenates was used for DNA extraction and a 1-μL portion of DNA template was subjected to PCR amplification using 106/107 and hdc-r/hdc-f primers as described above.

VI. *PCR Detection of Gram Negative Histamine-Producing Bacteria*

For the detection of *E. aerogenes* 05 or *R. ornithinolytica* 06 by PCR in pure culture, the overnight cultures of *E. aerogenes* 05 or *R. ornithinolytica* 06 were diluted, suspended in fresh TSBH broth (10^2 CFU/mL) and incubated at 30°C for 24 h. PCR assay, bacterial growth and histamine production of *E. aerogenes* or *R. ornithinolytica* were determined at 2, 4, 6, 8, 10, 12 and 24 h. Viable cell counts were determined by pour-plating 1-mL aliquots of the cultures serially diluted as necessary in duplicate on TSA plates and incubating at 30°C for 48 h. The amount of histamine in the culture broth was measured by HPLC as described above. One milliliter aliquots of the cultures were used for DNA extraction and a 1-μL portion of DNA template was subjected to PCR amplification using 106/107 and hdc-r/hdc-f primers as described above.

For the detection of *E. aerogenes* 05 or *R. ornithinolytica* 06 by PCR from spiked marlin homogenates, a block of freshly caught marlin fish was purchased from a commercial processor. The block was aseptically processed on a vertical laminar flow hood in our laboratory. The outer surface of the block was cut away to remove any potential cross-contamination from the surface to the inner muscle. A 30-g portion of internal muscle tissue was blended with 120 mL of 0.1% peptone water in a sterile blender. The homogenates were artificially contaminated with overnight cultured and serially diluted *E. aerogenes* 05 or *R. ornithinolytica* 06 (10^2 log CFU/g) and then incubated at 30°C for 24 h. PCR assay, bacterial growth and histamine production of *E. aerogenes* or *R. ornithinolytica* were determined at 2, 4, 6, 8, 10, 12 and 24 h. Viable cell counts were determined

Table 2. PCR results for bacterial strains isolated from Taiwanese foods to detect PCR specificity

Strain	PCR result and amplicon size	Histamine secretion (ppm)	Source
Gram (+)			
Staphylococcus capitis TS19-1	+, 367	1260	Mustard pickle
S. capitis TS19-2	+, 367	854	Mustard pickle
S. capitis TS19-3	+, 367	940	Mustard pickle
S. pasteuri MH16-1	+, 367	20	Mustard pickle
S. pasteuri D10-1	+, 367	20	Douchi
S. capitis D15-2	+, 367	600	Douchi
S. capitis D16-1	+, 367	287	Douchi
Lactobacillus sp. 30a	+, 367	500	ATCC 33222
Non-histamine producers (4 strains)**	—	ND	Mustard pickle and douchi
Gram (−)			
Enterobacter aerogenes 02	+, 534, 709	989	Sailfish fillet
E. aerogenes 05	+, 534, 709	1321	Sailfish fillet
E. aerogenes 05-1	+, 534, 709	1003	Sailfish fillet
E. aerogenes 05-2	+, 534, 709	1218	Sailfish fillet
E. aerogenes DM-2	+, 534, 709	562	Dried milkfish
Raoultella ornithinolytica 06	+, 534, 709	1870	Sailfish fillet
R. ornithinolytica 061	+, 534, 709	1225	Sailfish fillet
R. ornithinolytica 06-1	+, 534, 709	1221	Sailfish fillet
R. ornithinolytica 06-2	+, 534, 709	1237	Sailfish fillet
R. ornithinolytica MF6	+, 534, 709	1243	Dried milkfish
R. ornithinolytica 3-2	+, 534, 709	593	Tuna dumpling
R. ornithinolytica 3-3	+, 534, 709	593	Tuna dumpling
R. ornithinolytica 9-1	+, 534, 709	593	Tuna dumpling
R. ornithinolytica 40-1	+, 534, 709	595	Tuna dumpling
R. planticola 25-1	+, 534, 709	560	Tuna dumpling
Proteus vulgaris *	+, 534, 709	2000	Albacore
Morganella morganii *	+, 534, 709	2000	Albacore
E. aerogenes Ea10370	+, 534, 709	1287	ATCC 13048
M morganii Mm10760	+, 534, 709	1250	ATCC 25830
Non-histamine producers (6 strains)***	—	ND	Seafood (various samples)

* The histamine-forming bacteria were kindly provided by Dr. S. H. Kim.
** Non-histamine producers included *Bacillus* spp. (3 strains) and *Lactobacillus brevis* (1 strain).
*** Non-histamine producers included *Escherichia coli* (2 strains), *Vibrio parahaemolyticus* (1 strain), *V. alginolyticus* (1 strain), *Pseudomonas putida* (1 strain) and *Serratia liquefaciens* (1 strain).

by pour-plating 1-mL aliquots of the marlin homogenates serially diluted as necessary in duplicate on TSA plates and incubating at 30°C for 48 h. The amount of histamine in the marlin homogenates was measured by HPLC as described above. One milliliter aliquots of the marlin homogenates were used for DNA extraction and a 1-μL portion of DNA template was subjected to PCR amplification using 106/107 and hdc-r/hdc-f primers as described above.

RESULTS AND DISCUSSION

I. *Specificity of Primers*

The specificity results for the PCR assay tested against 8 gram-positive histamine-producing strains, 19 gram-negative histamine-producing strains and 10 non-histamine producing strains are presented in Table 1. The JV16HC/JV17HC primer set of gram-positive bacteria hdc gene

generated a single, typical PCR product of 367-bp for *Lactobacillus* sp. 30a (reference strain) and seven histamine producers, including *Staphylococcus capitis* (five strains) and *Staphylococcus pasturi* (two strains), were isolated from Taiwanese mustard pickle and douchi products. This primer failed to amplify the DNA of four gram-positive non-histamine producers (Table 2). Le Jeune *et al*.[20] reported that JV16HC/JV17HC primer set was successfully used to detect histamine-producing *Lactobacillus* 30a and *Clostridium perfringens* strains and was shown to be suitable for the detection of all histamine-producing lactic acid bacteria. In this study, the results suggested that the JV16HC/JV17HC primer was also suitable for the detection of histamine producer, *Staphylococcus* spp. Similarly, the 106/107 and hdc-r/hdc-f primer sets of gram-negative bacteria hdc gene generated 534-bp and 709-bp PCR products, respectively, for the two reference strains, *E. aerogenes* (ATCC 13048) and *M. morganii* (ATCC 25830), and two histamine producers, *Proteus vulgaris* and *M. morganii* provided by Dr. Kim[8]. All of 15 histamine producers, including *E. aerogenes* (five strains), *R. ornithinolytica* (nine strains) and *R. planticola* (one strain), isolated from Taiwanese seafood products (including sailfish fillet, dried milkfish and tuna dumpling) had clear PCR amplicons of 534 and 709 bp for 106/107 and hdc-r/hdc-f primer sets, respectively. Both primer sets did not amplify the DNA of six gram-negative non-histamine producers (Table 2). Takahashi *et al*.[21] reported that the PCR primers (hdc-f/hdc-r) amplify the hdc gene of gram-negative histamine-producing bacteria in fish samples and other sources. Strains of *M. morganii, R. planticola, E. aerogenes, Enterobacter amnigenus, Photobacterium damselae, Photobacterium phosphoreum, Hafnia alvei, Erwinia* sp. and *Proteus vulgaris* were positive using the PCR method proposed by Takahashi *et al*.[21]. However, De las Rivas *et al*.[22] found that the primers designed by Takahashi *et al*.[21] gave unspecific amplicons in some strains at the annealing temperature (52°C) for a multiplex PCR assay. They designed the 106/107 synthetic primers (Table 1) that were more specific than Takahashi's primers in the multiplex PCR assay conditions. This is not surprising, since Takahashi *et al*.[21] performed their uniplex-PCR assay without unspecific amplicons observed at a higher annealing temperature (58°C) than the optimized temperature for the multiplex assay (52°C). In this study, we demonstrated that histamine-producing gram-negative bacteria isolated from Taiwanese seafood products could also be successfully detected by primer set 106/107 and hdc-r/hdc-f. In addition, as we performed the PCR assay at a higher annealing temperature of 58°C instead of 52°C, no additional unspecific amplicon was observed in Figure 1 and 2 by using the hdc-f/hdc-r primer.

II. *Sensitivity of the PCR Detection for Gram-Negative Histamine-Producing Bacteria*

The results for the sensitivity of PCR assay for the detection of serially diluted *E. aerogenes* and *R. ornithinolytica* in TSB broth or fish homogenates using primer sets 106/107 and hdc-f/hdc-r were shown in Figure 1 and 2. *E. aerogenes* and *R. ornithinolytica* in TSB broth at levels of 10^6 to 10^8 CFU/mL were detected after the PCR amplification using the 106/107 primer (Figure 1A). The levels of *E. aerogenes* and *R. ornithinolytica* detected after the PCR amplification using the hdc-f/hdc-r primer were 10^5 to 10^8 CFU/mL in TSB broth (Figure 1B). However, the hdc gene was detected in *E. aerogenes* and *R. ornithinolytica* by the 106/107 primer PCR when the diluted bacterial concentrations were 10^7 to 10^9 CFU/g in fish homogenates (Figure 2A). In contrast, *E. aerogenes* and *R. ornithinolytica* in fish homogenates at levels of 10^6 to 10^9 CFU/g were detected after the PCR amplification using hdc-f/hdc-r primer (Figure 2B). Therefore, the minimum levels of detection of *E. aerogenes* or *R. ornithinolytica* after PCR amplification using hdc-f/hdc-r and 106/107 primers were 10^5 and 10^6 CFU/mL in TSB broth, and 10^6 and 10^7 CFU/g in fish homogenates, respectively. These results indicated that the hdc-f/hdc-r primer had higher sensitivity than the 106/107 primer for PCR amplification of gram-negative histamine producers, and the detection limit of the hdc-f/hdc-r or 106/107 primer in culture broth was 10-fold of that of fish homogenates. The lower sensitivity of hdc-f/hdc-r and 106/107 primers PCR shown in this study compared to the PCR of other bacteria between 10^2 to 10^3 CFU/mL in culture broth[27] is presumably because of the oligomixed and degenerated primers used to detect a wide range of histamine producers[21,22]. The lower sensitivity in fish homogenate compared to culture broth in the PCR assay might be due to the common inhibitors of PCR amplification including various components of body fluids and reagents encountered in clinical specimens (hemoglobin, urea and heparin), and food constituents (organic and phenolic compounds, glycogen, fat and Ca^{2+}) [28].

III. *PCR Detection of Gram-Negative Histamine-Producing Bacteria*

PCR detection assay, growth and histamine production of *E. aerogenes* in TSBH broth and marlin homogenates inoculated with *E. aerogenes* at a level of 2.0 log CFU/mL(g) were shown in Figure 3. The hdc gene was detected in TSBH broth by hdc-f/hdc-r and 106/107 primers PCR assay at 6 h and 8 h when the *E. aerogenes* counts were 5.95 log CFU/mL and 7.63 log CFU/mL, and histamine contents were 0 ppm and 10 ppm, respectively. A low level of histamine (10 ppm) was detected by HPLC after 8 h. The bacterial counts increased to 9.3 log CFU/mL as maximal after 12 h, while the histamine content was 729 ppm by HPLC (Figure 3A). Similarly, the hdc gene was detected in marlin homogenates by hdc-f/hdc-r and 106/107 primers PCR assay at 6 h and 8 h, respectively, when the *E. aerogenes* counts were 6.08 log CFU/g and 7.58 log CFU/g, respectively. A low level of histamine (13 ppm) was detected by HPLC after 8 h. Bacterial count and histamine content increased rapidly until about 9.4 log CFU/g and 1,010 ppm after 24 h, respectively (Figure 3B).

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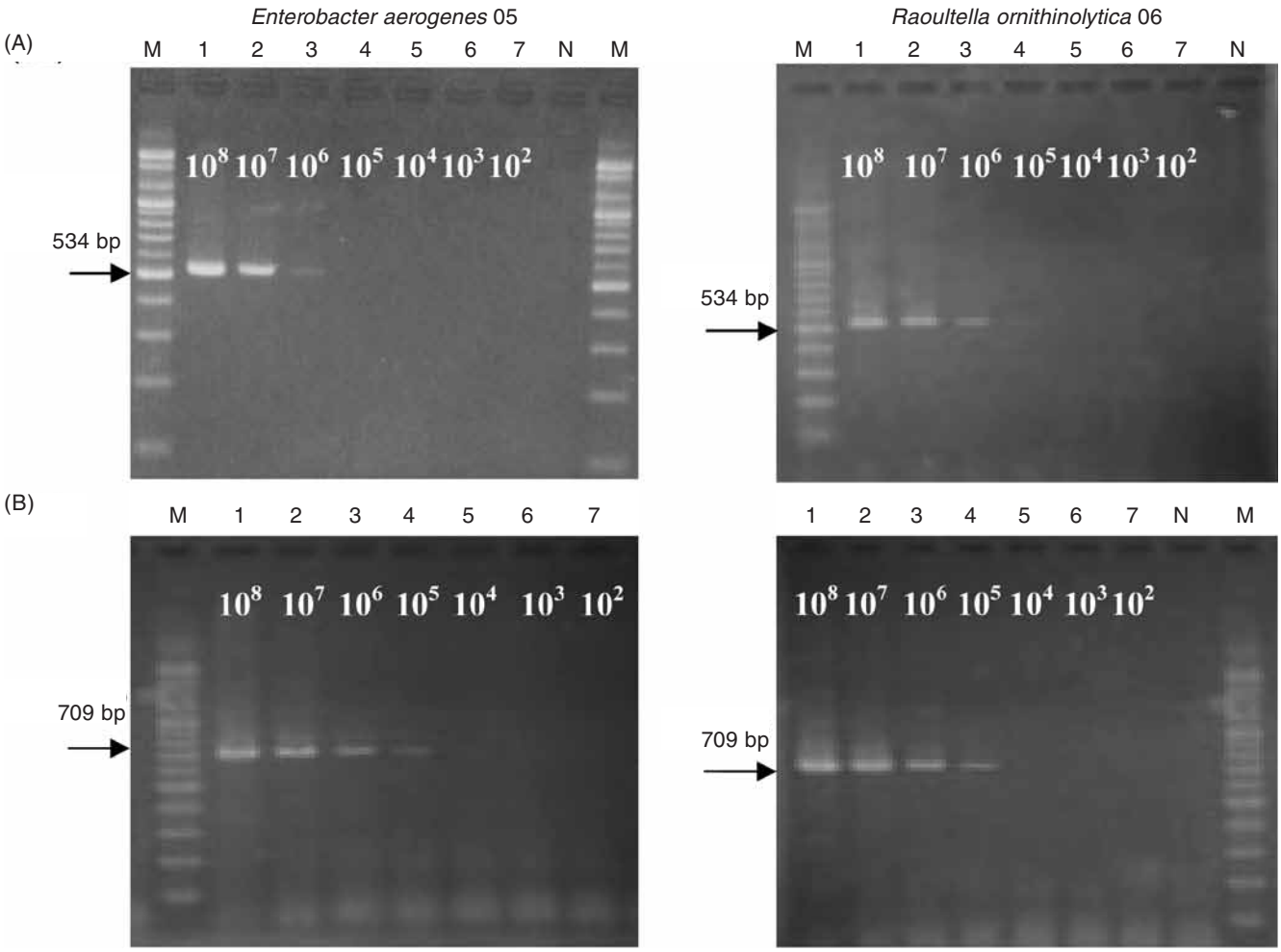

Figure 1. Sensitivity of PCR assay for the detection of serially diluted *Enterobacter aerogenes* 05 (left) and *Raoultella ornithinolytica* 06 (right) in TSB broth using primers 106/107(A) and hdc-f/hdc-r (B). Lane M, 100-bp ladder; lanes 1 - 7, 1×10^8 to 10^2 CFU/mL; lane N, non-spiked TSB used as a negative control.

PCR detection assay, growth and histamine production of *R. ornithinolytica* in TSBH broth and marlin homogenates inoculated with *R. ornithinolytica* at a level of 2.0 log CFU/mL(g) were shown in Figure 4. The hdc gene was detected in TSBH broth by hdc-f/hdc-r and 106/107 primers PCR assay at 6 h and 8 h, respectively, when the *R. ornithinolytica* counts were 5.92 log CFU/mL and 7.46 log CFU/mL, respectively. A low level of histamine (8 ppm) was detected by HPLC after 8 h. The bacterial counts increased to 9.26 log CFU/mL as maximal after 12 h, while the histamine content was detected at 716 ppm (Figure 4A). Similarly, the hdc gene was detected in marlin homogenates by hdc-f/hdc-r and 106/107 primers PCR assay at 6 h and 8 h, respectively, when the *R. ornithinolytica* counts were 6.12 log CFU/g and 7.61 log CFU/g, respectively. A low level of histamine (9 ppm) was detected by HPLC after 8 h. Bacterial count and histamine content increased rapidly until they reached about 9.15 log CFU/g and 1,000 ppm respectively after 24 h (Figure 4B).

In this study, *E. aerogenes* or *R. ornithinolytica* in TSBH broth or fish homogenates at levels of 5.92 to 6.12 log CFU/mL(g) were detected after PCR amplification using the hdc-f/hdc-r primer, which levels of 7.46 to 7.63 log CFU/mL(g) were detected after PCR amplification using the 106/107 primer (Figure 3 and 4). The hdc amplification using the hdc-f/hdc-r primer was detected 2 h earlier than HPLC detection of histamine in TSBH broth or fish homogenates. However, the detection of hdc amplification using the 106/107 primer and that of histamine by HPLC occurred at the same sampling time (Figure 3 and 4). Takahashi *et al.*[21] demonstrated that PCR amplification products using the hdc-f/hdc-r primer were detected from pure culture and tuna homogenates with viable counts of *M. morganii* higher than 10^4 to 10^5 CFU/mL(g). The difference could be due to the use of different species of microbial histamine producers and fish in the various studies. Many studies [8,13,29,30] have shown that detectable amounts of histamine accumulated only after the fish were decomposed or after aerobic plate counts reached a level greater than 10^7 CFU/g in fish. This was also confirmed in our study in which histamine content

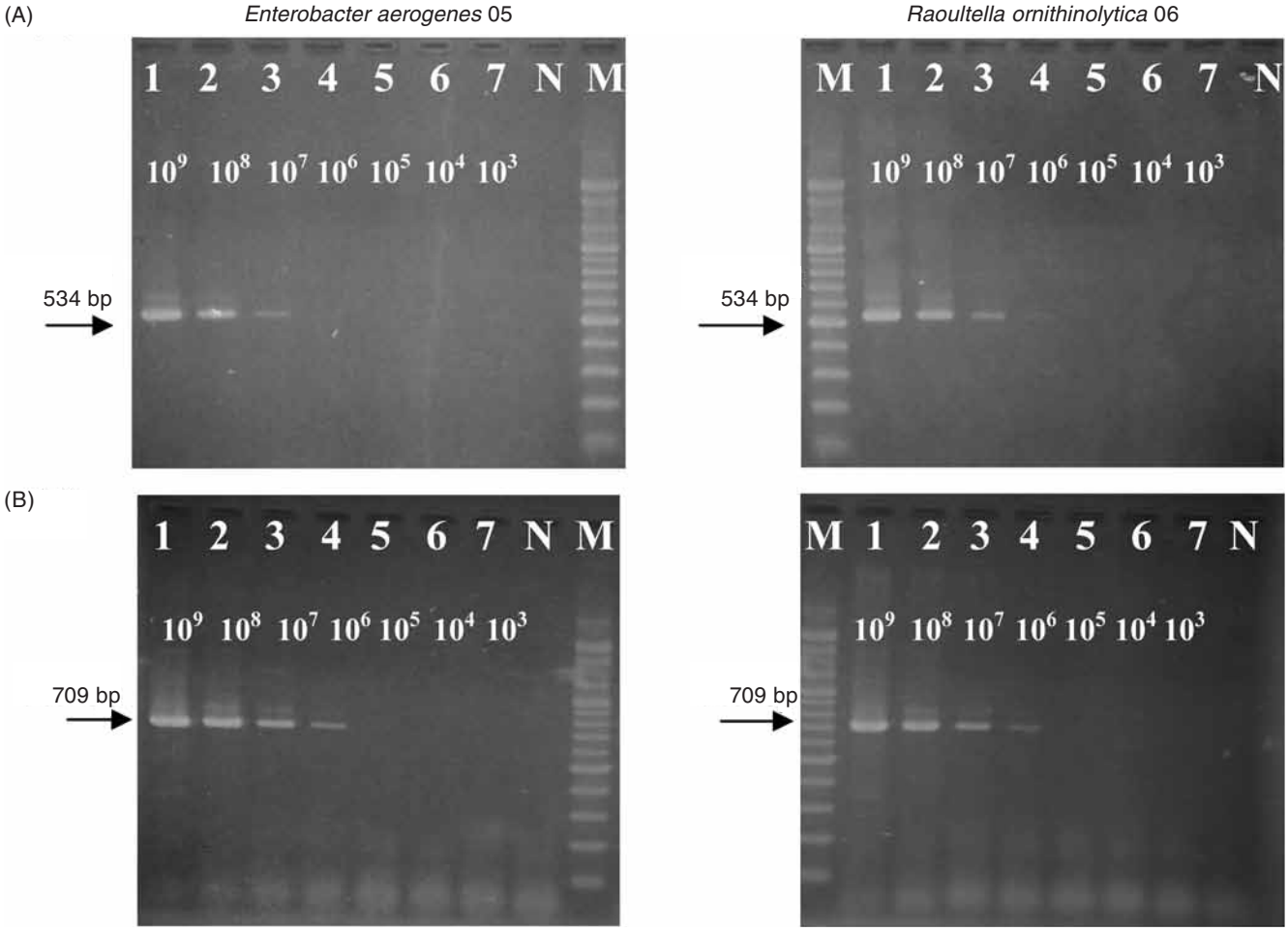

Figure 2. Sensitivity of PCR assay for the detection of serially diluted *Enterobacter aerogenes* 05 (left) and *Raoultella ornithinolytica* 06 (right) inoculated in marlin homogenates using primers 106/107 (A) and hdc-f/hdc-r (B). Lane M, 100-bp ladder; lanes 1 - 7: 1×10^9 to 10^3 CFU/mL; lane N, non-spiked marlin homogenate used as a negative control.

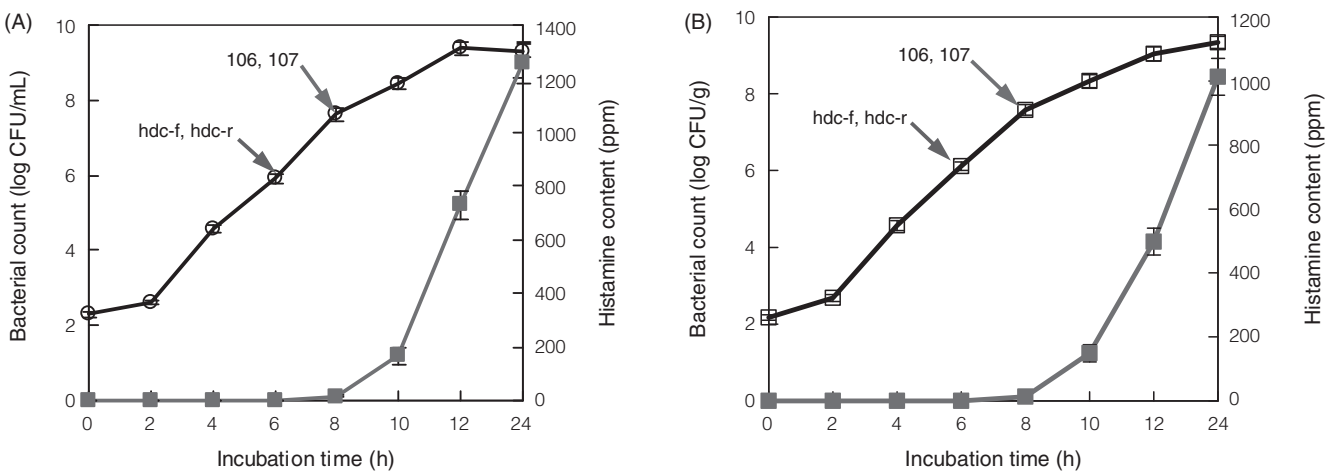

Figure 3. Growth, histamine production using HPLC method, and PCR detection assay for *Enterobacter aerogenes* 05 in TSBH broth (A) and marlin homogenates inoculated with *E. aerogenes* 05 (B) using primers 106/107 and hdc-f/hdc-r. Arrows indicate the sampling times at which PCRs became positive for the first time. Symbols: ■, histamine; □, *Enterobacter aerogenes* 05.

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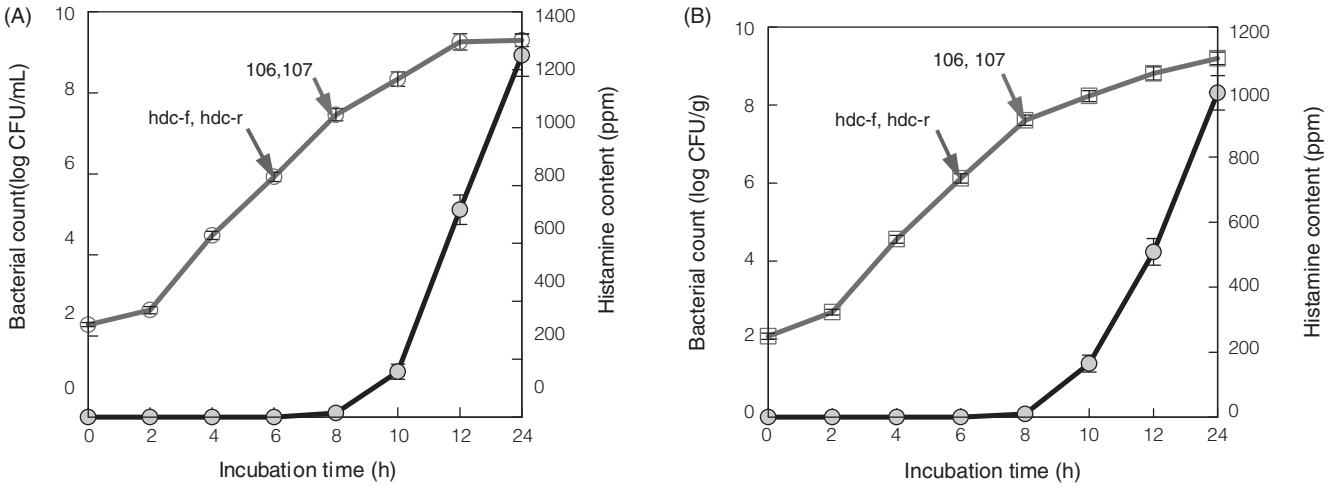

Figure 4. Growth, histamine production using HPLC method, and PCR detection assay for *Raoultella ornithinolytica* 06 in TSBH broth (A) and marlin fish homogenates inoculated with *R. ornithinolytica* 06 (B) using primers 106/107 and hdc-f/hdc-r. Arrows indicate the sampling times at which PCRs became positive for the first time. Symbols: ●, histamine; □, *Raoultella ornithinolytica* 06.

was detectable at levels of 7.46 to 7.63 log CFU/mL(g) after 8 h. Despite the low sensitivity of PCR in this study, it was still possible to detect gram-negative histamine producers via hdc-f/hdc-r primer PCR assay before histamine can be detected by HPLC.

CONCLUSIONS

This study showed that all histamine-producing strains isolated from Taiwanese foods produced a PCR amplification product according to the corresponding primer. In contrast, none of the non-histamine-producing stains produced an amplification product. The hdc-f/hdc-r primer seems to be more sensitive than the 106/107 primer in the detection of histamine-producing gram-negative bacteria based on sensitivity and spiked fish homogenates assay. The use of PCR method for the early and rapid detection of histamine-producing bacteria is important for preventing the accumulation of histamine in food products.

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