Original Article

SCREENING OF ANTIBIOTIC RESISTANCE GENES IN VIBRIO AND AEROMONAS SP. ISOLATED FROM THAI RICE-FERMENTED FISH PRODUCTS

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Abstract

Vibrio and Aeromonas sp. are genus of foodborne pathogens especially occurred in aquatic animals and environments. is also commonly isolates from aquatic organisms and environments, which can cause various symptoms in human, commonly gastro-intestinal diseases. Antibiotic resistance properties of Vibrio and Aeromonas sp. pathogens against multiple antibiotic drugs had reported. Thai traditional rice-fermented fish products including, pla-som (n = 15) and pla-chom (n = 15) are risk to contaminate with pathogenic bacteria. The study was aimed to identified Vibrio and Aeromonas sp. isolated from pla-som and pla-chom; and to evaluate antibiotic resistant properties and screen antibiotic resistant genes of isolated bacteria. According from colony, Gram □s staining and biochemical characteristics, Vibrio and Aeromonas sp. were identified. Antibiotic susceptibility of identified bacteria were determined against fifteen antibiotic discs. Screening of antibiotic resistance genes of Vibrio and Aeromonas sp. were also evaluated. All of isolated bacteria were possessed antibiotic resistance properties and multiple antibiotic resistances, which were occurred in V. vulnificus (n = 3), V. mimicus (n = 1), V. harveyi (n = 3) and A. hydrophila (n =3). Mostly of antibiotic resistance genes including vhhP2, tl and rpoS genes, were reserved in V. vulnificus isolates. V. parahaemolyticus and V. harveyi were carried tl and rpoS genes; and vhhP2 gene, respectively. Therefore, no antibiotic resistance genes were determined from V. mimicus, V. cholerae and A. hydrophila isolates. V. vulnificus isolated from this product was represented the relationship between phenotypic and genotypic occurrences, and ampicillin, chloramphenicol and cefuroxime were common antibiotic resisted.

Keywords: Vibrio vulnificus, Vibrio sp., pla-som, multidrug resistance (MDR), Thai rice-fermented fish.

INTRODUCTION

According to Food Agriculture Organization (FAO), world aquaculture production constitutes 1145 million tons which included 821 million tons of fish production [1]. Vibrio is a genus of Gram-negative bacteria belonging to Vibrionaceae family, which commonly found in aquatic environments. There are living as common flora of aquatic animals especially marine life. Approximately twelve Vibrio species are foodborne pathogens including V. vulnificus, V. parahaemolyticus and V. cholerae, which are common cause of diarrheal illness [2]. Particularly, V. vulnificus and V. parahaemolyticus are usually cause human infections, which are occur with the consumption of naturally contaminated raw, undercooked or cross- contaminated shellfish and fish [3-6]. Vibrio sp. are usually susceptible to commonly used antibiotics for human [7].

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Access this article online

Quick Response Code:

Website: www.pnrjournal.com

DOI: 10.47750/pnr.2022.13.04.199

How to cite this article: Kanittada T, Siriwipa P, Yuttana S, SCREENING OF ANTIBIOTIC RESISTANCE GENES IN VIBRIO AND AEROMONAS SP. ISOLATED FROM THAI RICE-FERMENTED FISH PRODUCTS, J PHARM NEGATIVE RESULTS 2022;13: 1419-1424.

V. parahaemolyticus and V. vulnificus are frequently antibiotic resistance with ampicillin, penicillin and tetracycline [8]. Several studies reported that majority of V. parahaemolyticus isolated from seafood, clinical specimens and environments are multi-drug resistance (MDR) including amoxicillin, ampicillin, bacitracin, carbenicillin, cefazolin, ceftazidime, cephalothin, colistin, gentamicin, penicillin, spectinomycin, and tobramycin [9-14]. Emergence of antimicrobial resistant V. cholerae are common cause of unsuccessful treatment [15]. Antibiotic resistance rate in V. cholerae is detected against sulfamethoxazole, nalidixic acid, trimethoprim, streptomycin, tetracycline and ampicillin, therefore there are susceptible to neomycin, azithromycin and ciprofloxacin [16, 17, 18]. Aeromonas sp. is a Gram-negative and non-spore-forming rod-shaped bacteria, which is also commonly isolates from aquatic organisms and environments [19]. This genus is including psychrophiles and mesophiles and can cause diseases in warm-blood animals (i.e., human) and cold-blood animals (i.e., fish), respectively [20]. There can cause various symptoms in human, such as gastroenteritis, soft tissue infection, septicemia and hepatobiliary tract infection [21]. Aeromonas foodborne outbreaks in various countries are associate with consumption of contaminated aquatic animals like Vibrio and A. hydrophila is most of causative bacteria. Psychrophilic Aeromonas including A. hydrophila, A. caviae and A. veronii biotype sobria are identified as human pathogens based on biochemical characteristics [22, 23]. Antibiotic resistance of Aeromonas pathogens against sulfonamide, trimethoprim, tetracycline, tetracycline, sulfonamide, trimethoprim and chloramphenicol had reported [24]. A. hydrophila isolates from aquatic animals are multiple antibiotic resistance including bacitracin, erythromycin, gentamicin, kanamycin, methicillin, nalidixic acid, neomycin, novobiocin, polymyxin-B, rifampicin, streptomycin, tetracycline, trimethoprim and vancomycin [25].

Pla-som and pla-chom are Thai traditional fermented fish, which is produced from large flesh/ whole of fish (pla-som) or tiny fish (pla-chom), sugar, salt and roasted rice (sometime contained with spices) and is fermented with natural microbial flora. Lactic acid bacteria are preserving this fermented-fish and vary on salt concentration [26]. According to the Thai Agricultural Standard, Thai fermented fish should meet in criteria of requirements including physical characteristics, salt content, food additives, and contaminants i.e., toxic metals and microbials. Traditional fermentation processes are risk to contaminate with pathogenic bacteria including Vibrio and Aeromonas sp. [27, 28]. The study was aimed to identified Vibrio and Aeromonas sp. isolated from pla-som and pla-chom; and to evaluate antibiotic resistant properties and screen antibiotic resistant genes of isolated bacteria, which were conducted. Our finding was concerning on horizontal resistance gene transferring through traditional Thai fermented fish. Hence, this data will be guide to local food manufacturers with and consumers on food safety awareness.

MATERIALS AND METHODS

Bacterial Culture and Isolation

Pla-som (n = 15) and pla-chom (n = 15) were purchased from six markets located in Bangkok, Thailand. Each fermented fish was weighed (25 g), suspended in 225 ml of 0.1% peptone water (Merck, Darmstadt, Germany) for enrichment (sample-to-broth ratio, 1:10) and inoculated in selective media (Himedia, India) including McConkey agar and Thiosulfate citrate bile salt sucrose (TCBS) agar at 37 □C for 24 hrs. Isolated colony was sub-cultured in tryptic soy agar (TSA) agar and incubated similar to selective media for biochemical and antibiotic susceptibility tests.

Identification of Pathogenic Vibrio and Aeromonas sp.

Bacterial colony isolate was characterized according by size, shape, convex, edge, color, and morphology. Each isolated colony was stained and identified by Gram □s negative staining and biochemical tests, respectively. Biochemical tests were included catalase, oxidase, citrate, methyl red, Voges-Proskuer test, oxidation-fermentation, lysine, indole, motility and bile esculin. String test and triple sugar iron (TSI) were used to identified Aeromonas sp. Fermentation of colony on TCBS agar and bacterial growing in 0%, 3%, 6%, 8% and 10% NaCl contained nutrient broth were used to identified Vibrio sp. Identification of bacterial isolates were interpreted according by Bergey□s Manual of Systematic Bacteriology and Clinical and Laboratory Standards Institute (CLSI) [29, 30].

Antibiotic Susceptibility Test

Pathogenic bacteria isolates were evaluated on antimicrobial susceptibility of by agar disc diffusion (Kirby-Bauer) method. Each isolated bacterial colony was sub-cultured in TSA agar, and was inoculated into 4 mL of sterile nutrient broth. Bacterial suspension was adjusted its density with 0.5 McFarland as standard. Then, bacterial suspension was spread over entirely surface of Muller Hinton (MHA) plate, which was dried by standing at room temperature. Fifteen of antibiotic discs (Difco, USA) used in susceptibility test were included amoxicillin/clavulanic acid (30 □g, 20/10 □g), ampicillin (10 □g), amikacin (30 □g), cefoxitin (30 □g), ceftazidime (30 □g), ceftriaxone (30 □g), cefuroxime (30 □g), chloramphenicol (30 □g), ciprofloxacin (5 □g), gentamicin (10 □g), imipenem (10 µg), meropenem (10 µg), piperacillin (30 □g) tetracycline (30 □g), trimethoprim/sulfamethoxazole (25 □g). Inhibition zone was clearing zone surrounded antibiotic disc and measured as diameter (mm). Each inhibition zone value was compared with CLSI reference and interpretated as susceptible (S), intermediate (I) and resistance (R) bacteria against tested antibiotic disc. For this study, intermediate antibiotic susceptibility bacteria were considered as antibiotic resistance and multi-drug resistance (MDR) bacteria were

defined as isolated bacteria is resisted three or more different antibiotic discs [31].

DNA Extraction and Screening of Antibiotic Resistance Genes

A single loopful of colony isolates of pathogenic Vibrio or Aeromonas sp. was extracted with QIAamp DNA mini-kit (Qiagen, Hilden, Germany). DNA templates of antibiotic resistance genes were amplified by multiplex PCR technique. Antibiotic resistance genes, primers and PCR conditions were summarized in Table 1. PCR fragment products were separated by agarose gel electrophoresis and interpreted of electropherogram according by reference methods [32, 33].

Table 1: Antibiotic resistance genes of Vibrio or Aeromonas isolates on multiplex PCR technique

Bacterial isolates	Gene	Primer sequence	Size (bp)	Reference
V. harveyi	vhhP2	Forward: CAG CTC CCC GTT TTT TAA ACC	157	Thongkao et al., 2016 [32]
		Reverse: CCA CCA TAT CCA TCG ATA TCT GTT		
V. parahaemolyticus	tl	Forward: AAA GCG GAT TAT GCA GAA GCA CTG	450	
		Reverse: GCT ACT TTC TAG CAT TTT CTC TGC		
V. vulnificus	rpoS	Forward: CAT GCG TGT TTC CTT GAT TC	273	
		Reverse: TCC ATA GCC TTT TTT CTA TTG G		
Aeromonas sp.	gyrB	Forward: GAA GGC CAA GTC GGC CGC CAG	198	Zhou et al., 2013 [33]
		Reverse: ATC TTG GCA TCG CCC GGG TTT TC		
	hly	Forward: GGC CGG TGG CCC GAA GAT ACG GG	597	
		Reverse: GGC GGC GCC GGA CGA GAC GGG		

RESULTS AND DISCUSSION

Vibrio and Aeromonas colonies were represented in TCBS agar and McConkey agar, respectively (Fig. 1). Vibrio and Aeromonas sp. were identified according from colony, Gram □s staining and biochemical characteristics. Vibrio sp. were further identified by ability of fermentation on TCBS agar and growth in vary of salinity (Fig. 2).

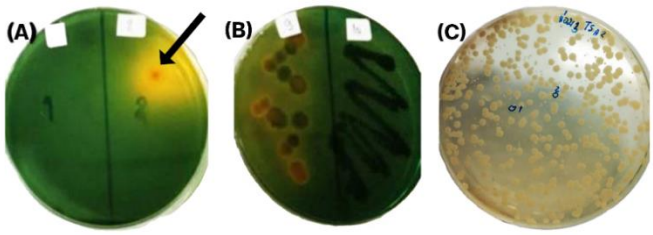

Fig. 1: (A) yellow-colored colony (sucrose fermented) on TCBS agar i.e., V. cholerae; (B) green-colored colony (non-fermented sugar) i.e., V. parahaemolyticus, V. vulnificus and V. mimicus; (C) Aeromonas colonies (non-fermented sugar) on McConkey agar

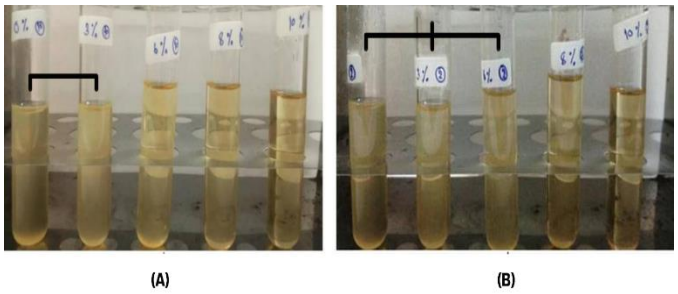

Fig. 2: Growth ability of Vibrio sp. in vary of salinity (a) low salinity growth (0-3%) i.e., V. cholerae and V. mimicus; high low salinity growth i.e., V. parahaemolyticus (1-8%) and V. vulnificus (1-6%)

Table 2: Antibiotic resistances of Vibrio and Aeromonas sp. isolated from Thai fermented fish

Sample/ Isolates	Antibiotic resistance pattern*	Resistance (%)	MDR (%)
Pla-som			
A. hydrophila (n = 2)	AMP-TE-IPM; AMP-TE	2 (100%)	1 (50%)
V. harveyi (n = 2)	AMP-CX-TE	1 (50%)	1 (50%)
V. mimicus (n = 1)	AMP-CX-C-CXM-TE	1 (100%)	1 (100%)
V. vulnificus (n = 1)	AMP-CTR-C-CXM-TE	1 (100%)	1 (100%)
Pla-chom			
V. cholerae (n = 3)	AMP	1 (33%)	-
V. vulnificus (n = 2)	AMP-AMC-C-CXM-TE-COT-IMP; AMP-C-CXM-COT-IMP	2 (100%)	2 (100%)
V. parahaemolyticus (n = 1)	AMP	1 (100%)	-
V. harveyi (n = 1)	CX	1 (100%)	-
A. hydrophila (n = 1)	AK-AMP-CAZ-PI-TE-COT	1 (100%)	1 (100%)

*Pattern of antibiotic drug resistance for each isolate and MDR was defined as bacterial resistant against three or more antibiotics

AK = amikacin; AMP = ampicillin; AMC = amoxiclav; CAZ = ceftazidime; C = chloramphenicol; CTR = ceftriaxone; CX = cefoxitin; CXM = cefuroxime; PI = piperacillin; TE = tetracycline; COT = co-trimoxazole; IPM = imipenem; MDR = multidrug – resistant bacteria

Some of pathogenic bacteria were isolated from Thai rice-fermented fish, including V. cholerae (n = 3), V. parahaemolyticus (n = 2), V. vulnificus (n = 3), V. mimicus (n = 1), V. harveyi (n = 3) and A. hydrophila (n = 3). Pathogenic Vibrio sp. and A. hydrophila isolated this product may out of standard according by Food and Agriculture Organization (FAO), which were concerned on food safety during fermentation process [34]. Mostly of salt-tolerate Vibrios and A. hydrophila can tolerated in salinity and acidity of these fermented fish products. However, organic acid and other metabolites were produced from lactic acid bacteria (LAB) including Lactobacillus, Lactococcus, Pediococcus, Streptococcus, Enterococcus and Weissella sp., can inhibited pathogenic bacteria [35, 36].

All of isolated bacteria were possessed antibiotic resistance properties and multiple antibiotic resistance, which were occurred in V. vulnificus (n = 3), V. mimicus (n = 1), V. harveyi (n = 3) and A. hydrophila (n =3) (Table 2). Antibiotic resistance genes including vhhP2, tl and rpoS genes, were reserved in all of V. vulnificus isolates and represented in Lane 3 (157, 273 and 450 bp), Lane 4 (450 bp), Lane 5 (273 bp); Lane 7 (273 and 450 bp), respectively (Fig. 3). V. parahaemolyticus isolate was carried tl and rpoS genes (Lane 6: 273 and 450 bp); and V. harveyi isolates were carried only vhhP2 gene (Lane 1 and 2: 157 bp). Therefore, no antibiotic resistance genes were determined from V. mimicus, V. cholerae and A. hydrophila isolates in this study (data not shown).

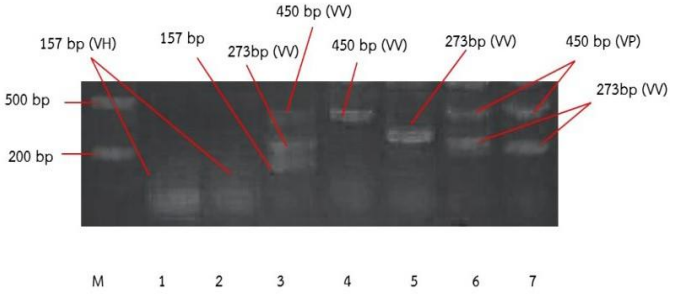

Fig. 3: Electropherogram genotyping for antibiotic resistances of Vibrio sp. Lane M = DNA (100 bp) ladder; Lane 1 and 2 = positive for vhhP2 gene (157 bp); Lane 3 = positive for vhhP2, tl and rpoS gene (157, 273 and 450 bp); Lane 4 = positive for tl gene (450 bp); Lane 5 = positive for rpoS gene (273 bp); Lane 6 and 7 = positive for tl and rpoS gene (273 and 450 bp); VH = V. harveyi; VV = V. vulnificus; VP = V. parahaemolyticus

As results, antibiotic resistance of V. vulnificus was represented the relationship between phenotypic and genotypic occurrences, that all of these bacterial isolates were resisted against five or more antibiotic including ampicillin, amoxiclav, chloramphenicol, cefuroxime, ceftriaxone, co-trimoxazole, imipenem and tetracycline. Ampicillin, chloramphenicol and cefuroxime were common antibiotics, which were resisted by V. vulnificus. In addition, imipenem resistance was commonly occurred in MDR bacteria. MDR property of V. vulnificus was high rate similarly to recently report of V. vulnificus distribution in Thai farmed fishes [37]. V. parahaemolyticus isolate was show only ampicillin resistance with non-genotypic occurred, which were implied that this isolate was pathogen without any virulence factor [38]. While, V. harveyi isolates were resisted only ampicillin, cefoxitin and tetracycline with

vhhP2 gene reserved. V. harveyi is common pathogen in aquatic animals, therefore, it is rarely cause of human diseases. As our finding, V. harveyi was carried own antibiotic resistance gene and its antibiotic resistance may cause from misuse of antibiotics to prevent spreading of diseases in many aquatic farming and excess antibiotic contained in environment [39, 40]. Cross-contamination of pathogens is possible during raw material handling and fermentation through containers [41]. In addition, lactic acid bacteria in fermented foods may also reservoirs of antibiotic resistance genes, which may transfer to pathogenic bacteria between food chain and environments [42]. Further study is need to conduct on fermentation and environments conditions of this products.

Conclusion

V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus, V. harveyi and A. hydrophila were isolated from Thai rice-fermented fish. All of isolated bacteria were possessed antibiotic resistance properties and V. vulnificus, V. mimicus, V. harveyi, and A. hydrophila were MDR bacteria. V. vulnificus was commonly resisted against ampicillin, chloramphenicol and cefuroxime, which was most reservoir of antibiotic resistance genes including vhhP2, tl and rpoS genes.

ACKNOWLEDGEMENT

We would like to sincerely thank to Faculty of Science and Technology, Suan Sunandha Rajabhat University were providing laboratory facilities. We are thankful students and technicians, Biology Program, Faculty of Science and Technology, Suan Sunandha Rajabhat University who support us as research assistants.

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