ความชุกและการดื้อยาต้านจุลชีพของเชื้อซัลโมเนลลาที่แยกได้จากหอยแครงในจังหวัดสุราษฎร์ธานี ประเทศไทย

Prevalence and Antimicrobial Resistance of Salmonella Isolated from Blood Cockles in Surat Thani Province, Thailand

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บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความชุกและการดื้อยาต้านจุลชีพของเชื้อซัลโมเนลลาที่พบในหอยแครงในธรรมชาติของจังหวัดสุราษฎร์ธานี ตัวอย่างหอยแครงถูกรวบรวมจากพื้นที่ต่างๆ ในปี พ.ศ. 2564 โดยแบ่งเป็นฤดูฝนและฤดูร้อน การตรวจหาเชื้อซัลโมเนลลาและการดื้อยาต้านจุลชีพของเชื้อซัลโมเนลลาใช้เครื่อง VIDAS® และ VITEK® 2 ตามลำดับ ผลการศึกษาพบเชื้อซัลโมเนลลาในหอยแครงที่เก็บตัวอย่างในฤดูร้อน (เมษายน) เท่านั้น ไม่พบเชื้อในฤดูฝน โดยมีความชุก 20% (4 จาก 20 ตัวอย่าง) การประเมินการดื้อยาต้านจุลชีพของสายพันธุ์ซัลโมเนลลาที่แยกได้ (n = 8) พบว่าเชื้อดื้อต่อยาอะมิคาซิน (6/8, 75%) เซฟฟาโลติน (8/8, 100%) ด็อกซีไซคลิน (8/8, 100%) เจนตามัยซิน (8/8, 100%) และเตตราไซคลิน (8/8, 100%) และความเข้มข้นของยาระดับต่ำสุดที่สามารถยับยั้งการเจริญของเชื้อ (MICs) มีค่าเท่ากับ ≥2, ≥2, ≥16, ≥1 และ ≥16 ไมโครกรัม/มิลลิลิตร ตามลำดับ เชื้อซัลโมเนลลาจำนวน 6 สายพันธุ์ที่แยกได้จากหอยแครงในการศึกษาครั้งนี้ดื้อยาต้านจุลชีพยาหลายชนิด (MDR) การศึกษานี้ให้ข้อมูลที่เป็นประโยชน์สำหรับการประเมินความเสี่ยงที่อาจเกิดขึ้นกับผู้บริโภค ซึ่งเป็นผลกระทบด้านสาธารณสุขที่สำคัญของประเทศไทย

คำสำคัญ: ความชุก, เชื้อซัลโมเนลลา, การดื้อยาต้านจุลชีพ, หอยแครง, จังหวัดสุราษฎร์ธานี

Citation:
Mala J, Rakthong P. Prevalence and antimicrobial resistance of *Salmonella* isolated from Blood Cockles in Surat Thani Province, Thailand. Health Sci J Thai 2023; 5(2): 48-56. (in Thai); https://doi.org/10.55164/hsjt.v5i2.258935

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Received: Aug 12, 2022; Revised: Dec 13, 2022; Accepted: Dec 23, 2022
https://doi.org/10.55164/hsjt.v5i2.258935

Abstract

This study was undertaken to investigate the prevalence and antimicrobial resistance of *Salmonella* in natural blood cockles from Surat Thani province. The research samples were collected from different areas over two seasons, dry and wet, in 2021. VIDAS® and VITEK® 2 compact systems were used for automated analysis. The results revealed that the prevalence of *Salmonella* was only found in the dry season, April (20%, 4 out of 20 samples) while all areas were not found *Salmonella* in the wet season. Antimicrobial resistance was evaluated in *Salmonella* strain isolated (n=8) and found that this pathogen was most frequently observed for amikacin (6/8, 75%), cefalotin (8/8, 100%), doxycycline (8/8, 100%), gentamicin (8/8, 100%) and tetracycline (8/8, 100%) with showed the minimum inhibitory concentration (MICs) were ≥2, ≥2, ≥16, ≥1 and ≥16 μg/ml, respectively. The six isolates demonstrated multiple drug resistance (MDR). This study provided useful information for the assessment of the possible risk posed to consumers, which has a significant public health impact in Thailand.

Keywords: Prevalence, *Salmonella*, Antimicrobial resistance Blood Cockles, Surat Thani province

Introduction

Blood cockle (*Tegillarca granosa* or the former specific name *Anadara granosa*) is one of the most valuable seafood species in the Bandon Bay, Surat Thani province, southern Thailand. It is not only significant in terms of its commercial value as food for human consumption but also remarkable as an edible species in the marine ecological food web[1]. However, it can readily harbor pathogenic microorganisms because of their filter-feeding nature and also the microbe laden habitat they inhabit[2]. Consumption of cockles poses a serious threat to public health when contaminated with pathogenic bacteria[3-4]. The most common pathogens associated with seafood-borne diseases are *Vibrio*, *Salmonella*, *Shigella* and *Clostridium botulinum*, respectively[5].

Salmonella is a rod-shaped, Gram-negative, oxidase negative, nonspore forming and predominantly peritrichous enterobacterium. It is also an important causative agent for food-borne diseases in humans who consumed aquaculture products[4-5]. *Salmonella* species have been reported and recognized as one of the leading causes of food-borne illness, causing diarrheal diseases and enteric fever that may be complicated by extra-intestinal infections, such as bacteremia, meningitis and osteomyelitis[6]. The incidence of salmonellosis caused by the consumption of contaminated seafood is a primary concern of public health agencies in many countries[4].

In Thailand, Epidemiological Surveillance Report Form (Report 506), food poisoning, food-borne disease and food-borne intoxication are classified as caused by microorganisms including *Salmonella* spp., *Vibrio parahaemolyticus*, *Staphylococcus* sp., *Clostridium perfringens* and unknown species. In 2020, Surat Thani Provincial Public Health Office has reported the food poisoning situation were a total of 10,693 patients which was the age group 0-4 years (1,862 cases), followed by the age group 25-34 years (1,694 cases) and 15-24 years (1,651 cases). Most of patients were employees and students at percentage of 60.10 and 27.59, respectively[7].

In recent years, antimicrobial resistance (AMR), particularly multidrug-resistance (MDR) strains, has become a major public health issue[4]. Excessive usage of antibiotics in the agriculture system resulted in the development and emergence of drug resistant seafood pathogens such as drug-resistant *Salmonella* strains[8]. Since antibiotics are widely used for growth promotion and disease treatment[9]. In AMR research from several publications always reported data as minimum inhibitory concentration (MIC) which is the lowest concentration of an antibiotic that prevents visible growth of pathogenic bacteria. Historically, most studies on the prevalence and characterization of antimicrobial resistance in *Salmonella* have focused on isolates from clinical and veterinary sources and other food products (meat and poultry). Information on the potential role of aquaculture food products especially blood cockles is very limited. Thus the aims of this study were to investigate the prevalence and antimicrobial resistance in blood cockles in Surat Thani province, Thailand.

Methods

Sample collection

Natural blood cockles were collected from Bandon Bay, Surat Thani province. The three most productive coastal areas and breeding grounds, Kanchanadit District (KD), Phunphin District (PP) and Tha-Chang District (TC) stations (Figure 1) were selected for this study. Global Positioning System (GARMIN model: GPSMAP 76CX) was used to mark the location of sampling points in coastal areas.

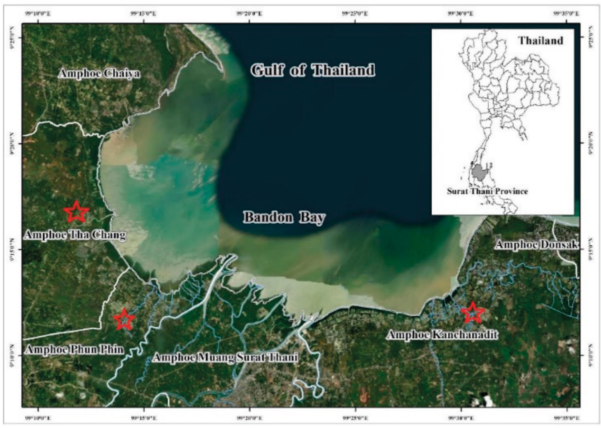

Figure 1 The sampling area in Bandon Bay, Surat Thani province

Samples were collected over two seasons, dry and wet, in 2021. There was a dry season during February to April, followed by a long wet season during May to January in Surat Thani province, based on the level of rainfall[10]. The sampling procedure was taken into account the suitability and number of samples used in the study to ensure statistical reliability. At each the station, blood cockles were collected 5 times for covering all seasons. Samples collected in January, August and November were used to represent those of wet season. Sample obtained in February and April were used to represent those of dry season. Eight to 12 individual blood cockles were pooled to get the total of 25 g of blood and whole-body soft tissues for microbiological analysis. At each station, two pooled sample were prepared for each time of collection. At PP station, the samples of January, August, November and April were not available. Moreover, blood cockles were also not received in November from TC station. Finally, twenty pooled blood cockle samples were obtained for further analysis. Samples were Animal Care and Use were carried out under animal use license number U1-04100-2559.

Microbiological analysis

A 25 g of natural blood cockles were weighted and the contents were extracted using a sterile 225 ml of Buffered Peptone Water. Each sample was homogenized by using a stomacher (Seward, UK) for 1 min at 250 rpm (twice) and incubated at 37 °C for 16-22 h. Following, samples were taken for 1 ml and placed in 6 ml of SX2 Broth (*Salmonella* Xpress) and incubated again at 41.5 °C for 22-26 h. After that, samples were analyzed by VIDAS® (bioMérieux, France) based on the specificity of antigen and antibody. A positive response in the immunoassay was surface streaked onto Salmonella Shigella (SS) agar for the isolation. Up to three colonies per isolation culture were confirmed with VITEK® 2 compact (bioMérieux, France).

Antimicrobial Susceptibility Testing

An antimicrobial susceptibility test was performed on VITEK® 2 compact (bioMérieux, France). The ID-GN and AST-GN 97 cards were used. To inoculate the VITEK-2 card, *Salmonella* isolates obtained from subculture on agar plates and overnight incubation were used to perform to make a bacterial suspension and adjusted to a McFarland standard of 0.5. At this point, the VITEK® 2 Compact cards were inoculated following the manufacturer's instructions. In brief, the confirmed *Salmonella* isolates were tested for susceptibility against 18 antimicrobial agents. The following 18 antimicrobial agents acquired from AST-GN97 cards were tested: amikacin, amoxicillin/clavulanic acid, ampicillin, cefalotin, ceftiofur, cefovecin, cefpodoxime, chloramphenical, doxycycline, enrofloxacin, gentamicin, imipenem, marbofloxacin, neomycin, nitrofurantoin, pradofloxacin, tetracycline and trimethoprim/ sulfamethoxazole, respectively. Antimicrobial susceptibility classified as resistant (R), intermediate (I) and susceptible (S) was interpreted in accordance with criteria established by the National Committee of Clinical Laboratory Standards (NCCLS)[11]. In addition, bacteria classified as either resistant or intermediate were defined as "non-susceptible" and those exhibiting resistance to at least one antimicrobial agent in three or more antimicrobial categories were defined as multidrug resistance (MDR).

Statistical analysis

All statistical analyses were performed and demonstrated as absolute frequencies and percentages.

Results

Prevalence of *Salmonella* in blood cockles

The prevalence of *Salmonella* contamination during the dry and wet seasons in differential three areas of Bandon Bay, Surat Thani province is shown in Table 1. A total of 20 samples were collected from January to November 2021, found that 4 of 20 samples (4/20, 20.0%) were positive for *Salmonella* detected by using VIDAS® (bioMérieux, France). However, the results only revealed that in the dry season was found the *Salmonella* contaminated in blood cockles while the KD and TC areas were found with the percentages of 20% (2/10) and 25% (2/8), respectively. In the case of the PP station, the natural blood cockles were not available. After that, four positive samples were characterized on Salmonella Shigella (SS) agar. Because *Salmonella* cannot ferment lactose but produce hydrogen sulfide (H_2S) gas in this media. Therefore, *Salmonella* colonies appeared colorless with black centers after appropriate incubation. Finally, eight single colonies were isolated for antimicrobial resistance analysis.

Table 1 Prevalence of *Salmonella* spp. in 20 samples of natural blood cockles in Surat Thani province, Thailand

Areas	Seasons	Months	No. of samples analyzed (n = 20)	Samples positive for *Salmonella* spp.	No. of strains isolated
KD	Wet	Jan	2	-	-
		Aug	2	-	-
		Nov	2	-	-
	Dry	Feb	2	-	-
		Apr	2	2 (2/10, 20%)	4
TC	Wet	Jan	2	-	-
		Aug	2	-	-
		Nov	Not Detected	-	-
	Dry	Feb	2	-	-
		Apr	2	2 (2/8, 25%)	4
PP	Wet	Jan	Not Detected	-	-
		Aug	Not Detected	-	-
		Nov	Not Detected	-	-
	Dry	Feb	2	-	-
		Apr	Not Detected	-	-

Antimicrobial susceptibility

A total of eight isolated *Salmonella* strains were identified and determined for their antimicrobial susceptibility. The average times required to obtain an identification and susceptibility result by using the direct test applied to the VITEK® 2 compact system were 5.12 ± 0.32 h and 9.33 ± 0.49 h, respectively. This system provided a final identification and susceptibility result in <10 h for 80% of the samples tested (Table 2).

The VITEK® 2 system next-generation platform provides greater automation while increasing safety and eliminating repetitive manual operations. The innovative VITEK 2 microbial identification system includes an expanded identification database, the most automated platform available, rapid results and improved confidence, with minimal training time. As shown in Table 2, all positive samples were comfily identified as *Salmonella* species by ID-GN card which was shown a 98-99% probability and excellent identification.

Table 2 Summarization of *Salmonella* spp. strain isolated confirmation and antimicrobial resistance using VITEK® 2 system

Strain	GN card analysis			AST-GN97 card analysis
	Analysis time (h)	%Probability	Confidence	Analysis time (h)
TT-Apr1	5.85	99	Excellent identification	10.5
TT-Apr2	4.87	98	Excellent identification	9.02
TT-Apr3	4.85	98	Excellent identification	9.00
TT-Apr4	5.13	99	Excellent identification	9.18
TC-Apr1	5.24	98	Excellent identification	9.46
TC-Apr2	5.07	98	Excellent identification	9.23
TC-Apr3	4.95	99	Excellent identification	9.12
TC-Apr4	4.98	99	Excellent identification	9.15
	5.12 ± 0.32	95.50 ± 0.53		9.33 ± 0.49

In this study, *Salmonella* isolates were resistant to amikacin, cefalotin, doxycycline, gentamicin and tetracycline with the MICs of ≥2, ≥2, ≥16, ≥1 and ≥16 μg/ml, respectively. On the other hand, resistance to amoxicillin/clavulanic acid, ampicillin, ceftiofur, cefovecin, cefpodoxime, chloramphenicol, enrofloxacin, imipenem, marbofloxacin, neomycin, nitrofurantoin, pradofloxacin and trimethoprim/sulfamethoxazole were not observed for any isolates. In addition, six *Salmonella* strains isolated from KD and TC sampling areas during the dry season (April 2021) showed multidrug resistance (Table 3). Moreover, the proportion of isolates which was resistant to at least two antimicrobial drugs was 75%. This rate is much higher than the 5% prevalence of multidrug resistant *Salmonella* isolates found in U.S.-imported food from Asia[4]. The high resistance to tetracycline (≥16 μg/ml), doxycycline (≥16 μg/ml), amikacin (≥2 μg/ml), cefalotin (≥2 μg/ml) and gentamicin (≥1 μg/ml), observed in the present study was probably due to the use of antibiotic agents in aquaculture feed and/or which are present at therapeutic or sub-therapeutic levels to prevent bacteriosis.

Table 3 Antimicrobial resistance patterns among *Salmonella* spp. strains isolated from natural blood cockles using VITEK®2 system susceptibility card (AST-GN97 card)

Antibiotics	*Salmonella* spp. isolates (n=8)						CLSI Guidelines[b] (μg/ml)
	R[a]	MIC (μg/ml)	I	MIC (μg/ml)	S	MIC (μg/ml)	
Amikacin	6 (75%)	≥2	0	-	2 (25%)	8	4-64
Amoxicillin/Clavulanic acid	0	-	0	-	8 (100%)	≤2	2-16/1-8
Ampicillin	0	-	0	-	8 (100%)	≤2	2-16
Cefalotin	8 (100%)	≥2	0	-	0	-	-
Ceftiofur	0	-	0	-	8 (100%)	≤1	-
Cefovecin	0	-	0	-	8 (100%)	1	-

Table 3 Antimicrobial resistance patterns among *Salmonella* spp. strains isolated from natural blood cockles using VITEK®2 system susceptibility card (AST-GN97 card) (contiued)

Antibiotics	*Salmonella* spp. isolates (n=8)						CLSI Guidelines[b] (μg/ml)
	R[a]	MIC (μg/ml)	I	MIC (μg/ml)	S	MIC (μg/ml)	
Cefpodoxime	0	-	0	-	8 (100%)	≤0.25	-
Chloramphenical	0	-	1 (12.5%)	16	7 (87.50%)	4	2-16
Doxycycline	8 (100%)	≥16	0	-	0	-	-
Enrofloxacin	0	-	4 (50.00%)	1	4 (50.00%)	2	-
Gentamicin	8 (100%)	≥1	0	-	0	-	-
Imipenem	0	-	0	-	8 (100%)	≤0.25	1-8
Marbofloxacin	0	-	0	-	8 (100%)	≤0.5-1	-
Neomycin	0	-	0	-	8 (100%)	≤2	-
Nitrofurantoin	0	-	0	-	8 (100%)	≤16	-
Pradofloxacin	0	-	5 (62.50%)	0.5	3 (37.50%)	2	-
Tetracycline	8 (100%)	≥16	0	-	0	-	0.5-8
Trimethoprim/Sulfamethoxazole	0	-	0	-	8 (100%)	≤20 to 40	2-4/38-76

Note: [a]R: resistant, I: intermediate, S: susceptible, [b]Concentration ranges of selected antibiotics tested for food-borne disease according to CLSI guidelines

Discussions

Several studies reported a *Salmonella* prevalence of 7.4% in oysters in the United States and 29.7% in retail aquaculture products in Shanghai, China[(4)]. While the prevalence of *Salmonella* isolated in seafood products sold in retail markets in Bangkok, Thailand was indicated at 36%[(5)]. Similarity to Woodring *et at.* reported that 21% of *Salmonella* was found in uncooked seafood in Thailand[(12)] and *Salmonella* spp. were detected in 0.6% of the samples (mussels, oysters and clams)[(3)]. The differences in these prevalence rates might be caused by differences in sample types, collection methods and laboratory methodology, as well as in local environmental conditions. However, the high *Salmonella* positive rate in aquaculture products is particularly alarming. Hence, it is important to control *Salmonella* infection in the food production chain to prevent the contamination of aquaculture products.

Prevalence of *Salmonella* in dry season was higher than those of wet season. Bandon Bay is an estuary of many rivers from mainland for example Tapi and Phumduang river. Primary sources of coastal water contamination are urban runoff and sewers. Seasonal variation in the residence time of water mass in Bandon Bay might be a cause of higher prevalence of *Salmonella* in dry season. Water exchange time in dry season was 16-81 days, whereas those of wet season was 9-13 days[(13)]. Longer residence time of contaminated water in Bandon Bay causes longer exposure of blood cockles to bacteria, resulting in the detection of *Salmonella* in blood cockles of our study.

In this study, *Salmonella* isolates were resistant to amikacin, cefalotin, doxycycline, gentamicin and tetracycline. These antibiotics are for the treatment of bacterial infection in human. They are also used legally and illegally in animals. Amikacin is injected into Koi fish to treat bacterial infection. Doxycycline was given to baby tilapias to prevent infection during transportation[14]. Doxycycline, gentamicin and tetracycline are recommended for treatment of bacterial infection in small animals. According to the widely used of antibiotics in animals, there was a report of the detection of doxycycline residues in tilapia collected from a farm[14]. These antibiotics are widely available in the market and can be bought without prescription by health professionals and veterinarians.

A study by Maka and coworkers indicated that *Salmonella* was common resistant to nalidixic acid (52.8%) followed by tetracycline (32.1%), ampicillin (28.3%) streptomycin (28.3%) and sulphonamides (26.4%), respectively[9]. Another study by Mengist and coworkers reported the resistance of *Salmonella* to ampicillin (100%)[15], while Giacometti and coworkers reported the resistance to streptomycin (43%), ampicillin (40%) and tetracycline (36%)[8]. In 2022, Rama and coworkers also exhibited that *Salmonella* isolated from farms were frequently resistant to tetracycline (76%) and streptomycin (70%)[16].

The widespread use of antibiotics in human and veterinary medicine is well known, but indiscriminate overuse may give rise to the development of drug resistant strains of *Salmonella*. The development and accumulation of resistance to antibiotics in pathogens is a major issue in public health. It is generally accepted that, in developing countries, some drug-resistant *Salmonella* are of animal origin and acquire their resistance in animals before being transmitted to humans through the food chain[4]. The widespread use of antibacterial substances in human and animal therapies, as well as in aquaculture, has resulted in resistance to antimicrobial substances. The present study highlights the level of antimicrobial resistance in *Salmonella* associated with aquatic food products.

Antibiotic resistance is a threat to food security and human health. Many infections are harder to treat and conventional antibiotics are less effective. Antibiotic resistance leads to longer hospital stays, higher medical costs and increase mortality[17]. Moreover, antibiotic resistance not only affects human health, but also affects human gut flora. Resistance genes of clinical relevant are found in normal flora. However, the transfer direction of mobile genetic elements of resistance genes between the commensal gut flora and the pathogens is uncertain. The interplay between human, animals and environments is important in antibiotic resistance.

Bandon Bay is one of the most productive coastal areas and breeding grounds of blood cockles in Thailand. Blood cockles from Surat Thani province have been transported to all over Thailand for years by wholesalers and retailers. Duangjan and Choengthong reported that blood cockles were purchased by wholesalers and then resold at Mahachai Market, Samut Sakhon[18]. After that they were distributed to many provinces in northeast and middle regions of Thailand. Blood cockles were also sent to many provinces in southern Thailand. Logistic systems contribute in the distribution of antibiotic resistance *Salmonella* throughout the country.

The lack of blood cockle samples was a limitation of this study. We could not obtained samples from November from TC station. Not only TC station, but also PP station. The samples from January, August, November and April were not available. Blood cockles are sensitive to freshwater and generally cannot survive when water salinity is very low. The occurrence of heavy rain and flood crisis in 2021 caused a high amount freshwater runoff and flowed into the bay. Resulting in a low salinity of coastal sea water and finally the death of blood cockles in both TC and PP stations.

According to our results, blood cockles may serve as reservoirs for antibiotic-resistant *Salmonella*. Seasonal monitoring of *Salmonella* and its antibiotic resistance should be conducted regularly. Such information is required in order to establish science-based public health policies and to develop effective intervention strategies to ensure the safety of our food supplies. Eating well-cooked blood cockles should be promoted. Public relations on the spread of antibiotic resistance pathogens in animals and environments by using area-based information can be a powerful method for encouraging all levels of societies to participate the prevention of misuse and overuse of antibiotics. Communicating to all stakeholders in Surat Thani province by using our study can enhance public consciousness of people.

In conclusion, our findings demonstrate that *Salmonella* was found in natural blood cockles during dry season, especially in April 2021 in Surat Thani province, Thailand. The prevalence of *Salmonella* was 20% (4 out of 20 samples). Many of the *Salmonella* isolates were resistant to multiple antimicrobial agents and most frequently resistant to amikacin, cefalotin, doxycycline, gentamicin and tetracycline, respectively. The surveillance and monitoring systems on *Salmonella*, other food-borne pathogens and their antibiotic resistance profiles are a crucial step for the prevention and control the spread of antimicrobial resistance pathogens.

Acknowledgement

This research was financially supported in part by a cooperative agreement FDA-C0-2563-11627-TH from the National Science and Technology Development Agency (NSTDA).

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