Malaysian Journal of Microbiology, Vol 18(3) 2022, pp. 338-343
DOI: http://dx.doi.org/10.21161/mjm.211233

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

SHORT COMMUNICATION

Preliminary investigation of multiple antibiotic-resistant bacteria isolated from blood cockles (*Anadara granosa*) and green lipped mussel (*Perna viridis*)

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Received 13 July 2021; Received in revised form 13 February 2022; Accepted 1 March 2022

ABSTRACT

Aims: Bivalve molluscs are filter feeders that tend to accumulate and concentrate any suspended particles or bacteria from the water environment. Although they have been proven to accumulate heavy metals, investigation on the presence of antibiotic-resistant bacteria is still lacking. Therefore, this study examines the occurrence of antibiotic-resistant bacteria isolated from bivalve shellfish, namely, blood cockles (*Anadara granosa*) and green-lipped mussel (*Perna viridis*) purchased from markets in Johor, Malaysia.
Methodology and results: Samples purchased were homogenized and then diluted. Viable cell count and bacterial isolation were performed using diluted samples followed by an antibiotic susceptibility test (ampicillin, ciprofloxacin and tetracycline) conducted on the pure isolates. The total viable count of bacterial colonies for cockle and mussel samples ranged from 1.1×10^7 to 4.4×10^9 CFU/mL and 1.2×10^9 to 4.3×10^9 CFU/mL. The numbers of colonies isolated from respective bivalves were 11 and 6. Generally, cockles isolates revealed higher resistance towards all three antibiotics at or above the Clinical and Laboratory Standard Institute (CLSI) threshold value. Meanwhile, mussel isolates showed full susceptibility to any ciprofloxacin concentration and tetracycline but exhibited resistance to ampicillin at a concentration exceeding the CLSI value. The number of drug resistance isolates in cockle and mussel samples decreased with increasing antibiotic concentration. The maximum number of antibiotics the mussel isolates were resistant to was two, whereas cockle isolates achieved three.
Conclusion, significance and impact of study: The outcome of this study concludes that some isolates from cockle and mussel samples can resist antibiotic concentration above the CLSI threshold value. Resistance of more than the CLSI threshold level revealed that these isolates could pose significant health risks especially when the bivalves are ingested raw or undercooked.

Keywords: Antibiotics, antibiotics resistant bacteria, bivalve molluscs, MAR index

INTRODUCTION

Antibiotic resistance is one of the issues gaining attention worldwide (Robinson *et al.*, 2016; Rousham *et al.*, 2018). An estimate of 700,000 people worldwide are suffering from antibiotic-resistant infections each year (Koch *et al.*, 2021). While antibiotic-resistant bacteria (ARB) was listed as one of the emerging environmental pollutants (Serwecińska, 2020), United States Centers for Disease Control and Prevention viewed ARB as a threat killing at least 35,000 people annually (CDC, 2019). However, very little attention is given to their occurrence, especially in the marine environment (Koch *et al.*, 2021).

Bivalve molluscs, including clams, cockles, mussels, oysters and scallops are excellent bioindicators in marine ecosystems owing to their ability to actively filter, retain and concentrate suspended particles, including free-living or particle-bound bacteria from their surrounding environment (Grevskott *et al.*, 2017). Since time-integrated methods are highly recommended for the detection of ARBs compared to the grab sampling method, employing bivalve molluscs as bioindicators are the most suitable (Moles and Hale, 2003). Several studies on the detection of ARB isolated from various species of bivalve molluscs worldwide have been reported. For instance, all the *Shewanella putrefaciens* strains (100%)

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

isolated from bivalve species in West Sea, Korea were resistant to cephalothin, vancomycin and ampicillin (Kang *et al.*, 2013) and all *Vibrio parahaemolyticus* strains extracted from oysters (*Crassostrea gigas*) were resistant to vancomycin, ampicillin and streptomycin (Kang *et al.*, 2018). *Clostridium difficile* strains isolated found in Mediterranean mussel (*Mytilus galloprovincialis*) and saltwater clams (*Ruditapes philippinarum* and *Chamelea gallina*) samples in North Adriatic Italian Sea were found non-susceptibility to erythromycin (23%), clindamycin (17%), rifampicin (8.8%) and moxifloxacin (10.6%) (Agnoletti *et al.*, 2019). Meanwhile, green mussels, blood cockles and oysters sold in Thailand markets contained *Campylobacter* spp. strains that resisted erythromycin (72.7-75%), nalidixic acid (30-40%) and ciprofloxacin (21.2-25%) (Soonthornchaikul and Garelick, 2009). Those *Vibrio* isolates found in green-lipped mussels from Phillipines revealed drug resistance towards ampicillin (37.8%), nalidixic acid (10.4%), tetracycline (10.4%) and co-trimoxazole (9.3%) (Tabo *et al.*, 2015).

In Malaysia, blood cockles (*Anadara granosa*) and green-lipped mussels (*Perna viridis*) are commonly raised and harvested commercially owing to their dense and fast growth. These bivalves are well-known to accumulate heavy metals (Azman *et al.*, 2012; Hadibarata *et al.*, 2012; Hossen *et al.*, 2015; Dabwan and Taufiq, 2016; Mahat *et al.*, 2018) but the knowledge on the presence of antibiotic-resistant bacteria is fairly limited (Al-Othrubi *et al.*, 2014; Sahilah *et al.*, 2014; Tang *et al.*, 2014; Othman *et al.*, 2018; Shahimi *et al.*, 2021). *Vibrio parahaemolyticus* strains isolated from cockles showed a variety of resistance to ampicillin, i.e ranging from 58.0% to 93.8% (Al-Othrubi *et al.*, 2014; Tang *et al.*, 2014), whereas *Vibrio alginolyticus* in a recent study only possess resistance of 12.5% (Shahimi *et al.*, 2021). Resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from both green-lipped mussels and blood cockles towards penicillin (100%), amoxicillin-clavulanate (85.7%) and amikacin (71.4%) were observed (Othman *et al.*, 2018). Similar to other researches worldwide, all the studies thus far only focused on specific bacterial species except one study (Ahmad *et al.*, 2014) that mapped the resistant bacteria in these bivalve molluscs. Emphasing only on specific bacterial species could lead to the tendency of overlooking other pathogenic antibiotic-resistant bacteria that could potentially harm the consumer when the molluscs are ingested raw or undercooked. Moreover, consumers may also be exposed to other pathogenic resistant species introduced during the transfer of the molluscs from the fishermen to the wet market sellers.

Therefore, this study aims to determine the antibiotic susceptibility profile of bacteria isolated from raw blood cockles (*Anadara granosa*) and green-lipped mussels (*Perna viridis*) purchased from local open-stall wet markets in Taman Universiti, Skudai, Johor, Malaysia. The information provided on the bacteria susceptibility to antibiotics in the Malaysia bivalve molluscs, thus, contributes to the awareness and precaution to be taken when handling and preparing for consumption.

MATERIALS AND METHODS

Chemicals and materials

Marine broth (MB) was obtained from BD, USA. Qrec Asia (Selangor, Malaysia) supplied sterile saline water. Solidified nutrient agar (NA) and Muller-Hinton Agar were acquired from Merck (Darmstadt, Germany). Ampicillin, ciprofloxacin and tetracycline were acquired from Santa Cruz Biotechnology (Carlifornia, United States).

Sample preparation

One (1) kg of raw blood cockle and green mussel samples were purchased from randomly selected open-stall markets in Taman Universiti, Skudai, Johor. Each sample was kept in a separate plastic bag, transported in ice and processed immediately upon reaching the laboratory. 10 g of soft tissue (edible portion) removed from the shellfish were homogenized and then transferred into 90 mL MB followed by their incubation at 37 °C for 24 h. Then, serial dilutions ranging from 10^{-1} to 10^{-7} were prepared using sterile saline water (0.85%) (Qrec Asia, Malaysia). Incubated shellfish and MB mixtures were transferred to a sterile test tube and gently swirled for a few minutes. Aliquot of 100 µL of each dilution was spread on sterile Petri dishes containing solidified NA using a sterile glass spreader. Plates were then placed upside down in an incubator at 37 °C for 24 h. After the incubation, different well-isolated colonies were picked, streaked and incubated on new NA plates until pure cultures were obtained. Each colony was isolated based on pigmentation and morphological appearance and sub-cultured twice to ensure purity (Supplementary Information Table S1).

Viable cell counting

Bacterial colonies were quantified using the spread plate method where 0.1 mL of diluted samples was pipetted and spread evenly on the agar plates following by their incubation at 37 °C for 24 h. Only plates between 30 and 300 colonies were counted. The colony-forming unit per mL (CFU/mL) was determined using the equation (1).

$$\text{CFU/mL} = (\text{No. of colonies} \times \text{Dilution factor})/\text{Volume of culture solution} \quad (1)$$

Antibiotic susceptibility test

Antibiotic susceptibility test was performed according to the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1959). Briefly, the medium was prepared according to the manufacturer's instruction, autoclaved at 121 °C for 15 min and allowed to partially cool before pouring into sterile Petri plates in a laminar flow cabinet. Pure cultures isolated were then evenly streaked on the solidified medium with sterile swabs. The prepared antibiotic discs containing ampicillin, tetracycline or ciprofloxacin at a concentration ranging from 1 µg to 50 µg were aseptically

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 338-343
DOI: http://dx.doi.org/10.21161/mjm.211233

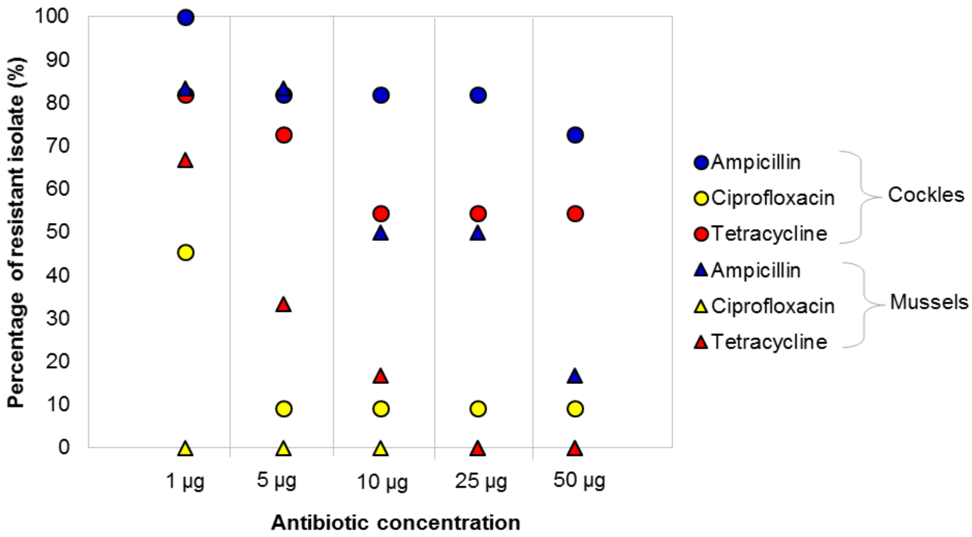

Figure 1: The percentage isolate resistant to antibiotics in green-lipped mussel and cockle samples.

placed on both the swabbed and unswabbed (for control purposes) plates and further incubated at 37 °C for 24 h. The size of the inhibition zone formed around the discs was recorded and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2017).

RESULTS AND DISCUSSION

The population density of bacterial colonies from cockle samples were found to be lower than the mussel samples. The bacterial counts in cockle samples ranged from 1.05×10^7 to 4.40×10^9 CFU/mL, while the count in green-lipped mussel samples ranged between 1.23×10^9 and 4.30×10^9 CFU/mL. The counts were higher than those observed in a previous study (Ahmad *et al.*, 2014). This difference might be due to several factors such as the feeding behaviour, original geographical source where the bivalve mollusks were collected, the practice of post-harvest and hygiene standards used during handling, transportation and storage of the bivalve mollusks samples (Letchumanan *et al.*, 2015). Only 11 and 6 isolates were successfully isolated from and blood cockle and green-lipped mussel samples, respectively.

Figure 1 illustrates the distribution of antibiotic susceptibility profile of bacterial isolates from cockles and mussels at different antibiotic concentrations. All cockle isolates were resistant to 1 µg and 81.8% were not susceptible to 5, 10, 25 µg of ampicillin. About seventythree (72.7) % of cockle isolates resisted to 50 µg of ampicillin. In the case of green-lipped mussels, the isolates resistant to 1 and 5 µg ampicillin were 83.3% and 50% susceptibility were observed at a concentration of 10 and 25 µg. Only 16.7% of the isolates showed resistance to 50 µg of ampicillin. It can be observed that the majority of the bacterial isolates from both cockles and mussels showed resistance to ampicillin with concentrations exceeding the concentration threshold set by CLSI, which is 10 µg (CLSI, 2017). Interestingly, the number of isolates resistant towards ampicillin were higher in cockle samples as compared to mussel samples. Compared with a previous study (Ahmad *et al.*, 2014), the percentage of cockle (40-88%) and green-lipped mussel (70.5-75.9%) isolates that were not susceptible to ampicillin of 10 µg was quite similar with those acquired in this study. Isolates for both bivalves displaying resistance to higher than the CLSI threshold concentration signify that the current dose used for treatment may not be sufficient if infected by them.

The potency of tetracycline was higher than that of ampicillin based on a lower percentage of the resistant isolates for both mussel and cockle samples. 66.7%, 33.3% and 16.7% of the mussel isolates demonstrated drug resistance to below 1, 5 and 10 µg of tetracycline, respectively, and none can be detected at concentration of 25 µg and 50 µg. The observation in cockle isolates was akin to the ones in ampicillin. Isolates resistant to 1 µg and 5 µg were 81.8% and 72.7%, respectively, whereas 54.5% of the isolates were immuned to a concentration above 10 µg. The findings illustrated by mussel and cockle isolates tested at highest tetracycline concentration (50 µg), however, opposed with previous studies conducted at CLSI threshold concentration of 30 µg. In previous studies, all MRSA isolates obtained from cockle samples and *Vibrio* species isolated from both bivalves showed full susceptibility at the threshold level (Tang *et al.*, 2014; Othman *et al.*, 2018; Shahimi *et al.*, 2021). Nonetheless, a variety of *Vibrio* isolates found in green-lipped mussels exhibited resistance ranging from 9% to 21% and *Vibrio parahaemolyticus* specifically has a resistance of 14% (Tabo *et al.*, 2015). This does not only imply that focusing on isolating certain pathogenic species may overlook other potential species that can pose threat to the consumer but also the resistance of similar bacteria species towards certain antibiotics may be location/country specific.

Resistant isolates percentage in cockle samples

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

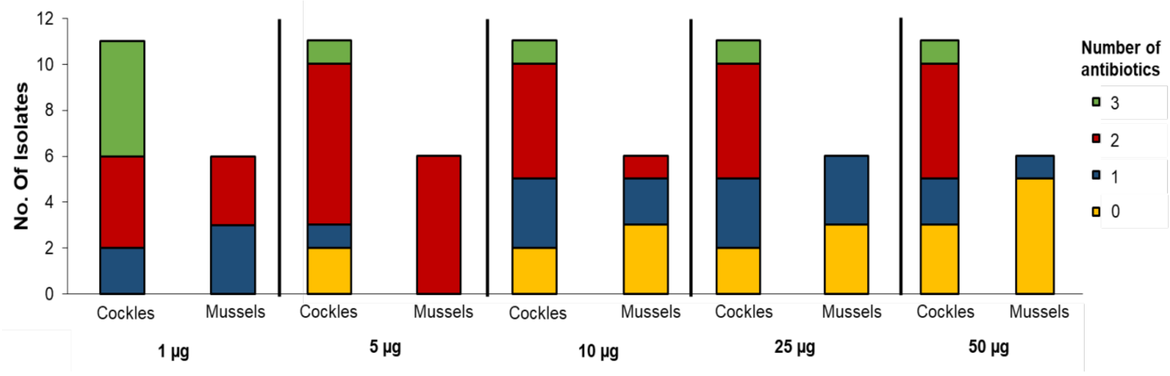

Figure 2: Number of isolates displaying multidrug-resistant at different antibiotic concentrations.

tested for susceptibility towards ciprofloxacin was the lowest among all the antibiotics investigated. The results were in good agreement with those previously studied (Ahmad *et al.*, 2014; Tabo *et al.*, 2015). Over foutyfive (45.5)% of the isolates were unsusceptible to concentration of 1 µg and the immunity of one particular isolate to ciprofloxacin of more than 5 µg making 9.1% of the total percentage. No resistant isolates can be detected in green-lipped mussels. This was expected as ciprofloxacin is the second-line antibiotic to be employed when first-line treatment, such as ampicillin and tetracycline, failed (Ministry of Health Malaysia, 2014). However, the results of cockle isolates is contrasting with previous study (Ahmad *et al.*, 2014) where cockle isolates were all susceptible to ciprofloxacin at the CLSI threshold of 5 µg. Meanwhile, green-lipped mussels collected from two different locations displayed 0% and 2.3% resistant isolates (Ahmad *et al.*, 2014). MRSA and *Vibrio parahaemolyticus* isolated from these two bivalves illustrated full susceptibility (Sahilah *et al.*, 2014; Tang *et al.*, 2014; Othman *et al.*, 2018). This, therefore, further substantiate the fact that some potential pathogenic bacterial species are ignored.

Multidrug-resistant demonstrated by both cockle and mussel isolates are shown in Figure 2. Typically, the multiple antibiotic resistant (MAR) index is used to assess the multidrug-resistant capability of a bacteria. MAR index lower than 0.2 indicated that the particular isolate was not exposed to the particular antibiotic studied (Chitanand *et al.*, 2010). However, MAR index could not be employed in this study because there were only three antibiotics tested. If MAR index are employed, the resistance of the isolates to one antibiotic will give an index of 0.33 causing misleading interpretation. Thus, the number of antibiotics resistant isolate was reported instead.

At a glance, the number of resistant isolates reduced with the increasing antibiotic concentration. The maximum number of antibiotics the mussel isolates resistant to were two, but cockle resistant isolates reached three. As the concentration of the studied antibiotics increased especially after 10 µg, the number of resistant isolates in green-lipped mussel samples decreased, but cockle isolates, remained nearly identical, especially those that were resistant to two or more antibiotics. The results were quite alarming due to the concentration were above the CLSI threshold level of ampicillin (10 µg), tetracycline (30 µg) and ciprofloxacin (5 µg). It is important to note that one of the isolates acquired from cockle samples can withstand all antibiotics above the CLSI designated concentration, suggesting that cockles may contain ineradicable bacteria. Nonetheless, a more in-depth study is required to identify the bacteria species prior to concluding if it is pathogenic.

CONCLUSION

The majority of the bacteria present in cockle and mussel samples was found to be resistant to ampicillin. While mussels samples showed high susceptibility towards tetracycline, cockle isolates resistance were relatively high. Some isolates can resist antibiotic concentration above the CLSI threshold level. Nevertheless, both bivalve isolates exhibited a relatively high susceptibility towards ciprofloxacin except for one particular isolate in the cockle that was resistant to all ciprofloxacin concentrations applied. As the concentration of antibiotics increased, the number of antibiotics the isolates resistant to decreased. The maximum number of resistant isolates was three and two for cockle and mussel samples, respectively. The outcome of this study concludes that there may be some resistant isolates that may be overlooked and thus, warrants a more in-depth study on the identification of the bacteria species to validate the pathogenicity of isolates of concern.

ACKNOWLEDGEMENTS

The authors would like to thank Universiti Teknologi Malaysia for the funding support under Tier 1 Research University Grant (Q.J130000.2522.17H15).

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SUPPLEMENTARY INFORMATION

Table S1: Morphology characteristics of bacterial isolates of blood cockles and green lipped mussels.

Bacterial isolates	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation	Optical property
a) Blood cockles								
4A	Circular	Entire	Flat	Punctiform	Smooth	Glistening	Non-pigmented	Translucent
4B	Circular	Entire	Raised	Small	Smooth	Dull	Non-pigmented	Translucent
4C	Circular	Entire	Raised	Small	Smooth	Dull	Non-pigmented	Opaque
4D	Circular	Entire	Flat	Punctiform	Smooth	Dull	Non-pigmented	Opaque
4E	Circular	Entire	Flat	Moderate	Smooth	Dull	Non-pigmented	Opaque
5A	Circular	Entire	Flat	Punctiform	Smooth	Glistening	Non-pigmented	Translucent
5B	Circular	Entire	Flat	Small	Smooth	Glistening	Non-pigmented	Opaque
6A	Circular	Entire	Raised	Punctiform	Smooth	Dull	Non-pigmented	Opaque
6B	Circular	Entire	Raised	Small	Smooth	Dull	Non-pigmented	Opaque
7A	Circular	Entire	Flat	Small	Smooth	Glistening	Non-pigmented	Opaque
7B	Circular	Entire	Flat	Punctiform	Smooth	Glistening	Non-pigmented	Translucent
b) Green mussels								
6A	Circular	Entire	Flat	Punctiform	Smooth	Glistening	Non-pigmented	Opaque
6B	Circular	Entire	Flat	Small	Smooth	Dull	Non-pigmented	Opaque
6C	Circular	Entire	Crateriform	Small	Smooth	Dull	Non-pigmented	Opaque
7A	Circular	Entire	Flat	Punctiform	Smooth	Glistening	Non-pigmented	Opaque
7B	Circular	Entire	Raised	Punctiform	Smooth	Dull	Non-pigmented	Opaque
7C	Irregular	Undulate	Flat	Moderate	Smooth	Dull	Non-pigmented	Opaque