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RESEARCH ARTICLE

Detection of pathogenic *Vibrio* species and antibiogram activity in Asian Seabass (*Lates calcarifer*) in Tumpat, Kelantan

Auzureen, A.M.Z.[1], Michael, M.S.[1], Mohamed, M.[1], Peng, T.L.[1], Fauzi, F.[1], Mohamad, N.F.A.[1], Ahmad, N.S.[1], C.W. Salma, C.W.Z.[1], Hamdan, R.H.[1*]

[1]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Pengkalan Chepa 16100 Kota Bharu, Kelantan, Malaysia
*Corresponding author: ruhil@umk.edu.my

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ABSTRACT

Some of *Vibrio* species is well known as pathogenic bacteria in aquaculture and the marine industry. Its infection is able to generate a massive outbreak and affect the fish population, especially for net caged fish such as seabass. This study was conducted to investigate the prevalence of *Vibrio* spp. isolated from seabass (*Lates calcarifer*) in Sri Tujuh Lagoon, Tumpat, Kelantan. Then, to determine the antibiotic resistance in *Vibrio* isolates. Polymerase chain reaction (PCR) was used to detect *Vibrio* species using specific primer VR169 and VR744 with estimation base pair size band, 597 bp and further identified by sequencing. On the other hand, antibiotic susceptibility tests were continued by using 13 types of antibiotics; kanamycin (K30), chloramphenicol (C30), neomycin (N10), ampicillin (AMP10), nitrofurantoin (F300), tetracycline (TE30), streptomycin (S10), norfloxacin (NOR10), ciprofloxacin (CIP5), nalidixic acid (NA30), gentamicin (CN10), doxycycline (DO30) and sulfamethoxazole (SXT100). As a result, 14 *Vibrio* isolates were identified, including *Vibrio fluvialis* (n=6), *Vibrio parahaemolyticus* (n=3), *Vibrio harveyi* (n=2) and each isolate for *Vibrio vulnificus*, *Vibrio alginolyticus* and *Vibrio* spp. The results showed that all isolates were sensitive to most antibiotics except ampicillin, neomycin and streptomycin. The MAR index value was ranging from 0 to 0.31. This study demonstrates the prevalence of *Vibrio* spp. in seabass and the report on multidrug resistance strains that could be of concern to the fish farmers. In addition, data from this study can be further used in fish disease management plans.

Keywords: *Vibrio* spp.; *Lates calcarifer*; antibiotic susceptibility test; multiple antibiotic resistance (MAR).

INTRODUCTION

Aquaculture industry is significantly expanded worldwide in the past decades (Stabili *et al*., 2022). In Malaysia, Asian Seabass is one of the most farmed marine fish, but it has been greatly affected by *Vibrio*sis (Ransangan & Mustafa, 2009). *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. funissii*, *V. fluvialis*, *V. damselae*, *V. mimicus*, *V. hollisae*, *V. cincinatiencis*, *V. harveyi* and *V. metchnikovii* are the most frequent *Vibrio* species transmit by the contamination of water or food (seafood) (Arunkumar *et al*., 2020). *Vibrio* species ubiquitous in marine or estuarine and capable of causing illness in human (Najiah *et al*., 2003; Jones, 2017). *Vibrio alginolyticus* was including with 11 non-cholerae *Vibrio* spp. and recognised as human pathogens (Jones *et al*., 2014). *Vibrio*sis were reported in marine life such as white leg shrimp (*Litopenaeus vannamei*), Atlantic salmon, oyster, seabass (*Lates calcarifer*) and grouper (Ransangan & Mustafa, 2009; Higuera *et al*., 2013; Jun *et al*., 2014; Lomelí-Ortega & Martínez-Díaz, 2014; Amalina *et al*., 2019). The outbreak may affect fish production and faced mortality. Usually, the *Vibrio* infection was related to raw consumption of seafood (Jones *et al*., 2014). Dahanayake *et al*. (2018) also mentioned the incidence of *Vibrio spp.* isolates committed with seafood-borne disease, like oysters that was preferred eating raw and undercooked in Korea.

Polymerase chain reaction (PCR) method is classified as sensitive, rapid and commonly used in screening the *Vibrio* spp. (Mohamad *et al*., 2019). This method provides advantages relative to and/or that complement standard microbiological culture-based methods (Jones *et al*., 2012). The previous studies of bacterial DNA marker mostly based on 16S rDNA sequences. The reason may be 16S rDNA sequences are easy to obtain and are homogeneous multiple copies in a bacterial genome (Yu *et al*., 2020).

Antibiotics use in aquaculture and livestock production by local farmers as it is the most affordable and the fastest way to prevent bacterial infection. *Vibrio parahaemolyticus* and *V. vulnificus* are identified as human pathogens of human seafood-borne infection (Jones, 2017). Prevention of bacterial infection may cause antibiotic abuse and be excessively used than suggested (Amalina *et al*., 2019). Seyfried *et al*. (2010) stated that antibiotic-impacted aquatic ecosystems, resistant bacteria and genetic material could be a medium of bacterial transportation and having contact with human and animal populations. Lee & Wee (2012) mentioned *Vibrio alginolyticus* in white leg shrimp were found resistant to lincomycin by 100% and sensitive to nitrofurantoin, furazolidone, tetracycline, florfenicol and oxolinate. Antibiotic susceptibility assay showed that *Vibrio harveyi* was effective towards oxytetracycline, nitrofurantoin, furazolidone, streptomycin, sulfamethoxazole, chloramphenicol,

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nalidixic acid and oxolinic acids, however, the usage of antibiotic was not recommended considered to its adverse effects (Ransangan & Mustafa, 2009). A study in 2013 showed that *V. alginolyticus* strain in cultured European sea bass from Aegean Region were resistant to ampicillin, bacitracin and streptomycin (Korun *et al*., 2013).

Pantai Sri Tujuh lagoon is adjacent to irrigation, a site where there have been numerous wastewater treatment overflows. Consequently, inputs from domestic sewage might be a cause of notable frequency of resistant bacteria detected in diseased seabass. Thus, the present study was carried out to isolate and identify the *Vibrio* species from diseased seabass. Then, evaluation of antibiotic resistance patterns of *Vibrio* spp. was done using 13 antibiotics.

MATERIALS AND METHODS

Bacterial isolation and identification

In 2019, a total of forty-seven (47) of diseases seabass (*Lates calcarifer*) (approximate weighing 300±0.1g) were sampled from the marine fish farm at Sri Tujoh Lagoon, Tumpat, Kelantan. Samples were purchased and immediately transferred to the laboratory in sterile condition. Diseased seabass showed clinical signs of exophthalmia, emaciation, skin darkening and body ulceration. Loopful of kidney, spleen and external lesion of the fish were streaked separately onto thiosulphate-citrate-bile-sucrose (TCBS) (Oxoid, England) and CHROMagar™ Vibrio (CHROMagar, France). The inoculated plates were incubated at 30°C overnight. The selected colonies were identified using Gram staining, oxidase, catalase and API 20E (BioMérieux, France).

Haemolytic activity assay

The bacterial isolates were inoculated on Columbia Blood agar with 5% sheep red blood (Oxoid, England). The plates were incubated at 30°C for overnight.

Molecular identification and DNA sequencing

DNA was extracted using NucleoSpin® Tissue kit (Macherey-Nagel, Germany). PCR amplification was conducted in 25.0µl reaction volumes containing 12.5µl 2x Green Master Mix (Promega, USA), 1µl forward and reverse primer, 5µl DNA template and 5.5µl nuclease-free water. The selective primer used in *Vibrio* spp. Detection genus 16S rDNA by Yong *et al*. (2006) as followed; VF169 (5'-GGATAACC/TATTGGAAACGATG-3') and VR744 (5'-CATCTGAGTGTCAGTG/ATCTG-3') with estimate base pair size, 597. Initial denaturation at 95°C for 4 min was followed by 35 cycles of amplification, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplification product was analysed by 1.5% agarose gel electrophoresis (Mupid-X, USA) using 1xTBE buffer, stained and visualised using Gel Doc™ E2 Imager (BioRad, USA). PCR products were purified using Gel/PCR DNA Fragment Extraction Kit (Geneaid, USA) and sent to Apical Sdn. Bhd. for further sequencing. The sequence information was compared with Genbank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibiotic susceptibility test

Antibiotic susceptibility test was determined using Kirby Bauer method (CLSI, 2015). Subsequently, a bacterial suspension with turbidity equivalent to 0.5 McFarland standard was prepared in a 0.85% NaCl solution. Using a sterile swab, the bacterial suspension was inoculated on Mueller-Hinton agar (Oxoid, England). The plates were incubated 30°C for 24 h. The results of inhibition zones were interpreted as sensitive (S), intermediate (I) and resistance (R) according to the standard provided by CLSI. The antibiotic discs were loaded onto MH agar. The antibiotic discs used in this study were kanamycin (K30), chloramphenicol (C30), neomycin (N10), ampicillin (AMP10), nitrofurantoin (F300), tetracycline (TE30), streptomycin (S10), norfloxacin (NOR10), ciprofloxacin (CIP5), nalidixic acid (NA30), gentamicin (CN10), doxycycline (DO30) and sulfamethoxazole (RL100) (Oxoid, England).

Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotics resistance (MAR) value of bacterial isolates was calculated based on the following formula;

$$\text{MAR index} = X / (Y \times Z)$$

Where X: Total cases of antibiotic resistance; Y: Total number of antibiotics used; Z: Total number of isolates. A MAR index value of less than or equal to 0.2 is considered to indicate that the animals are seldom or never exposed to antibiotics. If MAR index value was greater than 0.2 it is considered that the samples were isolated from high-risk source which has high rate of antibiotic exposed (Sarter *et al*., 2007).

RESULTS

Isolation and identification of *Vibrio* spp.

Fourty-seven isolates were presumptive *Vibrio* spp. based on colony morphology on TCBS and CHROM agar. After 24-hour incubation, yellow, green and creamy colonies appeared on TCBS agar showing *Vibrio* species bacterial presence. On CHROMagar *Vibrio*, mauve colour colonies represent *V. parahaemolyticus*, *V. vulnificus* has a typical appearance green blue to turquoise blue colonies while *V. alginolyticus* showed colourless creamy colonies (Figure 1). Gram staining showed negative with a short and curved rod shape. Each isolate was tested with oxidase test where the oxidase paper changed to blue-violet in 10 seconds. Figure 1 showed the observation of *Vibrio* colonies on CHROMagar *Vibrio* agar and TCBS agar.

Haemolytic activity assay

All the *Vibrio* isolates showed beta hemolysis on Columbia Blood agar with 5% sheep red blood (Oxoid, England).

Polymerase chain reaction and DNA sequencing

Fourteen out of 47 isolates were confirmed as *Vibrio* species using PCR (Figure 2). The details of each accession number were described in Table 1. The primers developed by Yong *et al*. (2006) were used in amplifying the rDNA from all selected bacterial samples. All fourteen *Vibrio* isolates were examined in BLAST and showed 98-100% homology sequences. Fourteen (14) *Vibrio* isolates were identified, including *Vibrio fluvialis* (n=6), *Vibrio parahaemolyticus* (n=3), *Vibrio harveyi* (n=2) and each isolate for *Vibrio vulnificus, Vibrio alginolyticus* and *Vibrio* spp.

Antimicrobial susceptibility test

Most of the *Vibrio* spp. showed 100% susceptibility to doxycycline, gentamicin, nalidixic acid, norfloxacin, tetracycline and nitrofurantoin. Meanwhile, 57% (n=8) of *Vibrio* spp. isolates were resistance to ampicillin. On the other hand, 50% (n=7) of the isolates were resistance to both neomycin and streptomycin. The resistance profile and MAR index of all the isolates is shown in Table 2. There was no significance difference of *Vibrio* isolates exposed to high contamination where the drug antibiotics were not often used.

Statistical analysis

Table 3 showed the comparison of inhibition zone between different antibiotics by using one-way ANOVA. There was a significant mean difference of the inhibition zone between antibiotic discs used at $p < 0.05$ [$F = 35.84$, $p = 0.000$]. Thus, from descriptive analysis, it can conclude that the highest mean (SD) of inhibition zone is TE30 (tetracycline) with 27.0 (2.25) (Table 3).

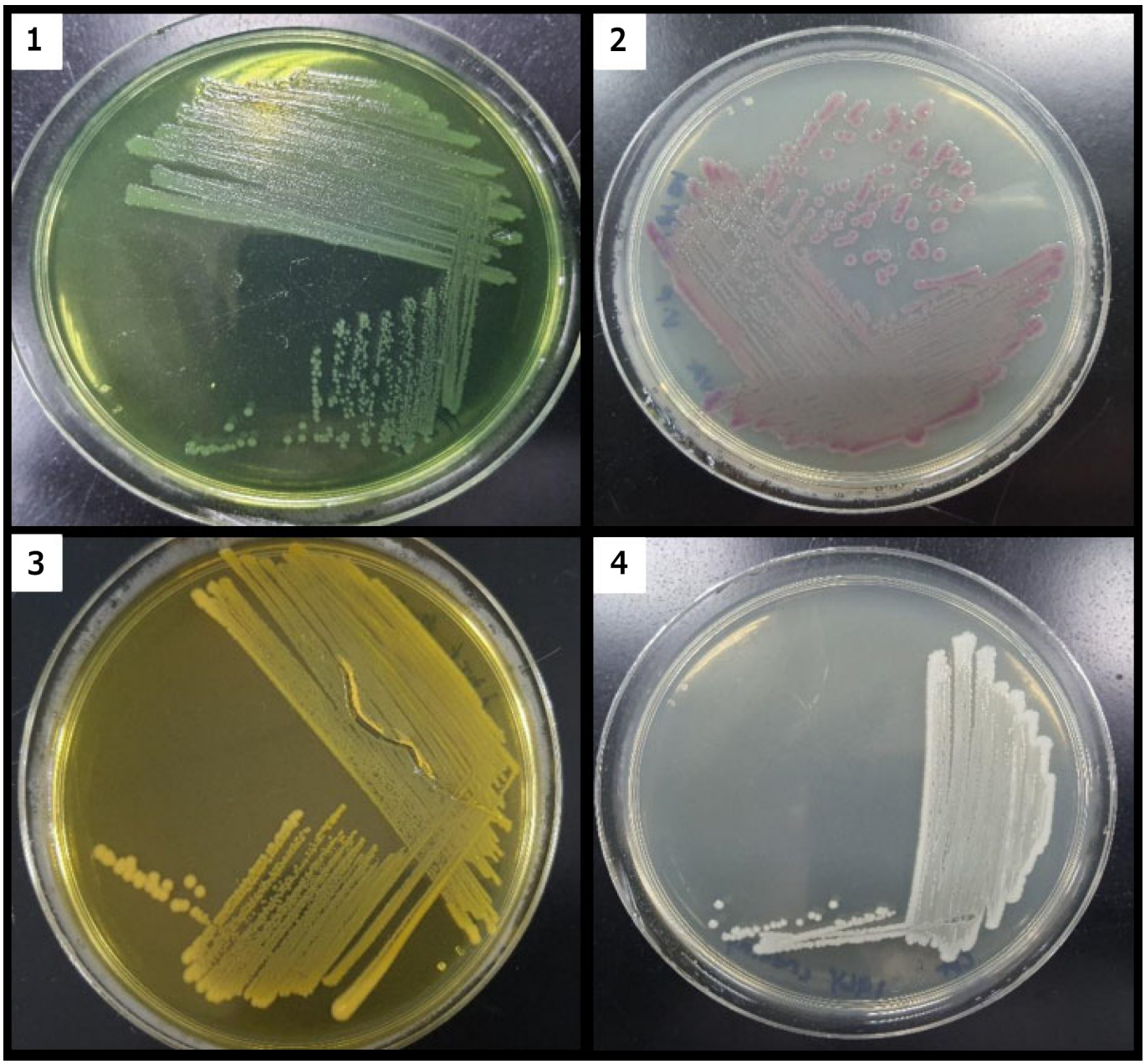

Figure 1. *Vibrio* spp. colonies on TCBS and CHROMagar *Vibrio* agar showed the (1) green colonies of *V. parahaemolyticus* on TCBS agar, (2) mauve colonies of *V. parahaemolyticus* on CHROMagar *Vibrio* agar, (3) yellow colonies of *V. alginolyticus* on TCBS agar, (4) creamy colonies of *V. alginolyticus* on CHROMagar *Vibrio* agar.

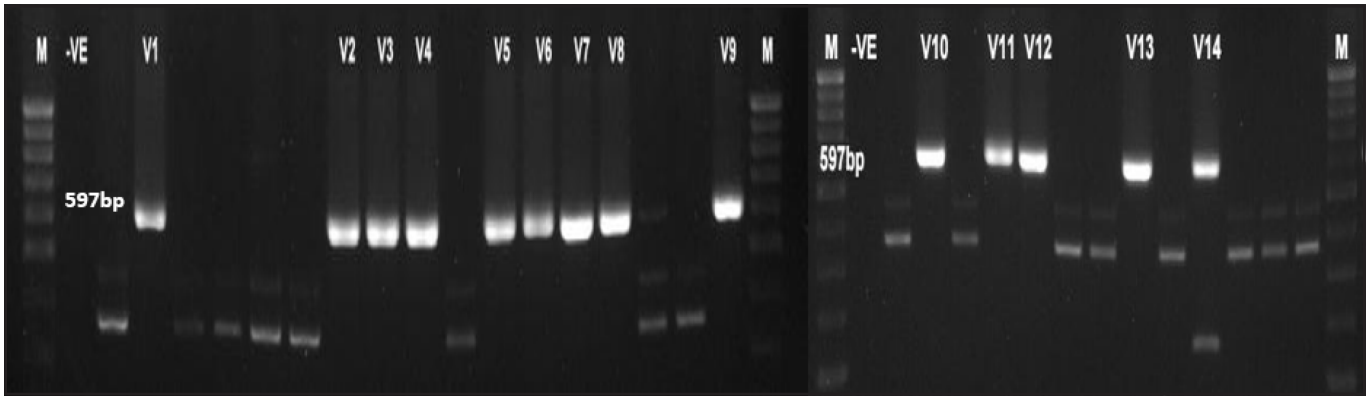

Figure 2. Fourteen (V1-V14) isolates of *Vibrio* spp. detected the expected band on polymerase chain reaction with length size, 597bp.

Table 1. Fourteen isolates were evaluated based on 16S rRNA sequencing of *Vibrio* spp. from disease seabass

Sample ID	16S rRNA sequencing	GenBank Accession number	% of Similarity
V1	*V. fluvialis*	KX710324	100
V2	*V. parahaemolyticus*	MK156400	100
V3	*V. harveyi*	KY003115	100
V4	*V. harveyi*	MK100328	100
V5	*V. fluvialis*	KX710324	100
V6	*V. fluvialis*		100
V7	*V. fluvialis*		100
V8	*V. vulnificus*	CP009261	100
V9	*V. parahaemolyticus*	MK156400	100
V10	*V. fluvialis*	KC210808	100
V11	*V. alginolyticus*	KJ371088	100
V12	*Vibrio* spp.	DQ991226	100
V13	*V. fluvialis*	KC210808	100
V14	*V. parahaemolyticus*	MK156400	100

Table 2. The antibiotic resistance profiles and MAR index value of *Vibrio* spp. isolates from seabass

Bacterial isolates	Resistance profiles	Resistance Patterns	MAR index
V2	N10, AMP10, SXT100, S10	I	0.31
V3	N10, AMP10, S10	II	0.23
V4	N10, S10	III	0.15
V5	AMP10	IV	0.08
V6	AMP10	IV	0.08
V7	AMP10	IV	0.08
V9	N10, AMP10, S10	II	0.23
V11	N10, AMP10, S10	II	0.23
V12	N10, S10	III	0.15
V14	N10, AMP10, S10	II	0.23

Table 3. Showed the comparison of inhibition zone between different antibiotics by using one-way ANOVA

Antibiotics	Variable	Results
K30	Mean (SD)	17.35 (3.02)
C10	Mean (SD)	25.50 (2.40)
N10	Mean (SD)	14.71 (2.52)
AMP10	Mean (SD)	9.92 (8.32)
F300	Mean (SD)	20.57 (1.98)
RL100	Mean (SD)	22.14 (3.75)
TE30	Mean (SD)	**27.00 (2.25)**
S10	Mean (SD)	12.00 (2.57)
NOR10	Mean (SD)	24.64 (3.71)
CIP5	Mean (SD)	25.14 (3.86)
NA30	Mean (SD)	26.78 (2.51)
CN10	Mean (SD)	18.14 (3.39)
DO30	Mean (SD)	24.85 (1.46)

DISCUSSIONS

Asian seabass has much potential marketing forces due to high demand from Malaysia and worldwide. Food and Agriculture Organization stated that the production of seabass has increased since 2000an, and Thailand is major producer followed by Indonesia, Malaysia and Taiwan (FAO, 2020). However, livestock development has urged the pathogenic infection and antimicrobial agents induced the multiple resistance reaction (Igbinosa, 2015). Antibiotics are generally used worldwide for treating diseases caused by pathogenic bacteria in humans and animals, including fish (Türe & Alp, 2016).

Thirty percent of *Vibrio* spp. infection detected in seabass from the fish farm in Sri Tujoh Lagoon, Tumpat, Kelantan. Six species of *Vibrio* were discovered e.g. *V. fluvialis*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Vibrio* spp. and *V. alginolyticus*. In this study, *Vibrio fluvialis* are the most *Vibrio* species isolates from Asian seabass. Zheng *et al*. (2017) stated that *V. fluvialis* was the unpopular emerging *Vibrio* pathogen with the least of the pathogenesis of mechanis. In contrary findings by (Luo *et al*., 2018) affirmed that *V. fluvialis* are the most *Vibrio* species infected seabass and able to survive in different environments. The outbreak of *Vibrio*sis caused by *V. alginolyticus* and *V. parahaemolyticus* have been reported in *Hippocampus kuda* (Xie *et al*., 2020); cobia (*Rachycentron canadum*) (Rameshkumar *et al*., 2017); white leg shrimp (Yen *et al*., 2020), Yesso scallop, (*Patinopecten yessoensis*) (De Silva *et al*., 2019), oysters (*Crassostrea virginica*) and clam, (*Mercenaria mercenaria*) (Jones *et al*., 2014), bloody clam (*Anadara granosa*), surf clam (*Paphia undulata*) and shrimps, *Penaeus* spp. (Malcolm *et al*., 2015). *Vibrio vulnificus* was always related with consumption of contaminated shellfish and exposure to poor marine environment (Chan *et al*., 1999). The contaminated sources of *V. vulnificus* were found by contaminated fish gear (Morimoto *et al*., 2009), ingestion of raw seafood (Jung *et al*., 2005), prick from dorsal fish fin (Chan *et al*., 1999). Human pathogenic *Vibrio* spp. (*V. parahaemolyticus*, *V. vulnificus* and *V. cholera*) were usually linked to climate change to seawater as could developing risk to human health (Hackbusch *et al*., 2020). Tan *et al*. (2020) reported that the majority of *V. parahaemolyticus* were not pathogenic to human because the lack of pathogenic *tdh* and *trh* genes.

In this study, all of the *Vibrio* isolates showed beta hemolysis on 5% sheep blood agar. Haemolytic activity assay showed the clear zone of colonies proven that it is identified as virulence factor in many pathogenic *Vibrio* including *V. parahaemolyticus* (Rattanama *et al*., 2012). Haemolysis activity causes the bacteria to attack the host defense by lysing the blood cells. The living bacteria will enter the bloodstream to the target organ and systemically spread to whole host body (Mulya *et al*., 2022).

The antibiotic susceptibility test was performed for all 14 *Vibrio* isolates from the seabass samples. The results show that 57.3% (n=8) of the bacterial isolates were resistant to ampicillin. Nevertheless, resistance to ampicillin is not uncommon in *Vibrio* spp. and has been used since 1960 (Venggadasamy *et al*., 2021). As a result of excessive and uncontrolled use of antibiotics in treating human disease and many agricultural practices, high incidences of ampicillin-resistant *Vibrio* spp. have been reported extensively in previous report from elsewhere (Kitiyodom *et al*., 2010; Yano *et al*., 2014; Kang *et al*., 2017; Amalina *et al*., 2019; Letchumanan *et al*., 2019; Li *et al*., 2020; Loo *et al*., 2020; Narayanan *et al*., 2020; Tan *et al*., 2020; Yen *et al*., 2020; Venggadasamy *et al*., 2021; Onohuean *et al*., 2022). Al-Othrubi *et al*. (2014) highlighted the increasing trend in the minimum inhibition concentration (MIC) of ampicillin from 64 µg/mL to 128 µg/mL in 2011 and 2013, respectively. Although ampicillin is not used in the management of Vibriosis, these findings are of great concern as it impedes the role of ampicillin in the empirical management of bacterial infections (Venggadasamy *et al*., 2021).

MAR indices were detected in this study ranging from 0.00 to 0.31, with 35.7% of the isolates having a MAR index value more than 0.2. Based on MAR index, isolates with indices higher than 0.2 are markers of high-risk sources, which may represent a potential human health risk (Sarter *et al*., 2007). In the present study, all isolates of *V. parahaemolyticus* showed MAR index ranging from 0.23 to 0.31, indicating that the strains were resistant towards three or more different antibiotics. Although the MAR index provides a good measure of the severity of antibiotic resistance in the samples, comparisons of MAR indices between studies are impossible to make due to the variation in the types of antibiotics tested and the total number of antibiotics used in individual studies (Venggadasamy *et al*., 2021). For an instance, comparison between the studies done by Narayanan *et al*. (2020) and Siddique *et al*. (2021) which demonstrates the highest MAR indices of *V. parahaemolyticus* isolates are 0.71 and 0.27, respectively. Narayanan *et al*. (2020) showed that the isolates were resistant to 17 out of 24 antibiotics, while Siddique *et al*. (2021), only resistant to four out of 15 antibiotics. In addition, antibiotic resistance level are influenced by the difference in geographical locations and selective pressures (Lesley *et al*., 2011; Tunung *et al*., 2012).

This present study showed tetracycline was listed as one of the susceptible antibiotics which is similar to Xie *et al*. (2020). The study stated that *V. harveyi* and *V. alginolyticus* were susceptibile to doxycycline and tetracycline. In addition, the present findings were inagreement with the study done by Amalina *et al*. (2019) who revealed that *Vibrio* spp. isolates from groupers (*Epinephelus* spp.) were susceptible towards tetracycline, streptomycin, erythromycin and bacitracin. Some authors stated that oxytetracycline, nitrofurantoin, streptomycin, sulfamethoxazole, chloramphenicol and nalidixic acid were susceptible against *Vibrio* spp., however, they considered the antibiotic usage were not supported as it may cause adverse effect especially in aquaculture (Ransangan & Mustafa, 2009; Rasul & Majumdar, 2017; Ibrahim *et al*., 2020).

CONCLUSION

This study was conducted to check the presence of *Vibrio* species diversity in farmed Asian seabass in Sri Tujuh Lagoon, Tumpat, Kelantan. The study found several species of *Vibrio* that are potentially pathogenic to Asian seabass as well as to human. Varying degrees of antimicrobial resistance were also observed among the isolated vibrios. Further investigations are needed to identify the risk factors that could trigger disease outbreaks in Asian seabass farms with *Vibrio* strains. Fish health surveillance programme should be implemented effectively to prevent disease outbreaks and will help to improve Asian seabass production in Malaysia.

Conflict of interest

The authors declare that they have no conflict of interests. This work was supported by Fundamental Research Grant Scheme (FRGS): FRGS/1/2019/WAB01/UMK/03/1 from The Ministry of Higher Education (MOHE), Malaysia.

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