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Prevalence, antimicrobial susceptibility and plasmid profiling of *Vibrio* spp. isolated from cultured groupers in Peninsular Malaysia

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Abstract

Background: Numerous prevalence studies of *Vibrio* spp. infection in fish have been extensively reported worldwide, including Malaysia. Unfortunately, information on the prevalence of *Vibrio* spp. in groupers (*Epinephelus* spp.) is limited. In this study, groupers obtained from nine farms located at different geographical regions in Malaysia were sampled for the presence of pathogenic *Vibrio* spp. and their susceptibility profiles against seven antibiotics.

Results: Out of 270 grouper samples, 195 (72%) were detected with the presence of *Vibrio* spp. *Vibrio communis* showed highest prevalence in grouper (28%), followed by *V. parahaemolyticus* (25%), *V. alginolyticus* (19%), *V. vulnificus* (14%), *V. rotiferianus* (3%), *Vibrio* sp. (3%), *V. campbellii* (2%), *V. mytili* (2%), *V. furnissii* (2%), *V. harveyi* (1%), *V. tubiashii* (1%), *V. fluvialis* (0.3%) and *V. diabolicus* (0.3%). Assessment on the antibiotic susceptibility profiles of the *Vibrio* spp. revealed that majority of the isolates were susceptible to tetracycline, streptomycin, erythromycin and bacitracin, but resistance to ampicillin, penicillin G and vancomycin. The mean MAR index of the *Vibrio* isolates was 0.51, with 85% of the isolates showed MAR index value of higher than 0.2. Results indicate that the *Vibrio* spp. were continuously exposed to antibiotics. Furthermore, the plasmid profiles of *Vibrio* spp. showed that 38.7% of the isolates harbored plasmid with molecular weight of more than 10 kb, while 61.3% were without plasmid. During curing process, *Vibrio* spp. lost their plasmid, but remained resistant to ampicillin, penicillin G, bacitracin and vancomycin while a few isolates remained resistant to erythromycin, streptomycin and tetracycline. The results suggested that the resistance to antibiotics in isolated *Vibrio* spp. might be due to chromosomal and plasmid borne.

Conclusions: This study demonstrates the prevalence of *Vibrio* spp. in groupers and the distribution of multidrug resistance strains that could be of concern to the farmers in Malaysia. In addition, data from this study can be further used in fish disease management plan.

Keywords: *Vibrio*, Grouper, Multiple antibiotic resistance, Plasmid, Chromosome, Malaysia

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Background

Aquaculture is a growing sector for food production, representing 47% of the total 171 million metric tons of fish supplies worldwide [22]. However, efficient fish production was hindered by health problems that cause mortalities and significant stock losses [6, 65]. Disease outbreaks following infections by pathogenic bacteria have been reported among various cultured marine fish such as grouper (*Epinephelus* spp.), pompano (*Trachinotus blochii*) and Asian seabass (*Lates calcarifer*) [3, 15, 44, 60].

Generally, molecular methods were used for the identification of bacteria species based on the specific molecular markers. *pyrH* genes is one of the common markers used in PCR and multi-locus sequence analysis (MLSA) to determine the taxonomic diversity of *Vibrio* spp. It is a housekeeping gene that encodes for Uridylate kinase (UMP kinase) and plays an important role for survival and growth of *Vibrio* [34]. Various studies have reported on the efficiency of the *pyrH* gene in identification and differentiation of *Vibrio* spp. [48, 54, 55, 63]. In addition, the *pyrH* gene has high discriminatory power at species level due to slight overlapped of intraspecies and interspecies distance [48, 59].

Antibiotics are the first line of treatment for bacterial infection and are frequently used by farmers, especially the wide spectrum antibiotics [8, 53]. Antibiotic is a chemical substance that has the capacity as therapeutic and prophylactic activities against growth of bacteria and is safe to the host [9]. In Malaysia, antibiotics are used both as prophylaxis and therapy in cultured fish. They are administered via feed additives or immersion baths [25].

Unfortunately, extensive use of antibiotics encouraged the emergence of antibiotic resistance bacterial strains [45]. According to Kumar et al. [32], occurrence of antibiotic resistance bacteria was common in areas where antibiotics were frequently used such as in outbreaks area. Letchumanan et al. [36] reported that the resistance level of pathogenic *Vibrio* spp. toward antibiotics used in aquaculture was increasing every year. In fact, some antibiotics have been reported to be ineffective in controlling bacterial pathogens [20].

When bacteria are overly exposed to antibiotics, they tend to acquire antimicrobial resistance genes, either via horizontal gene transfer or vertical gene transfer [57]. Thus, plasmid is one of the mediators that plays an important role in spreading of resistance genes since it consists most of the genetic determinants of antibiotic resistance. In fact, correlation between plasmid and antibiotic resistance among *Vibrio* spp. has been reported [37, 42, 68]. Similarly, several studies have shown that the antibiotic resistance genes were actually located in the bacterial chromosomal DNA [26, 40, 41].

Plasmid curing is a method that allows determination mode of antibiotic resistance mediation by eliminate bacteria plasmid. Chemical agents such as ethidium bromide (EtBr), sodium dodecyl sulphate (SDS) and acridine orange (AO) are commonly used to cure the plasmid [39, 50]. The mechanism involves inhibition of plasmid replication by intercalation of the chemical agent into the plasmid leading to unwinding of the super helical plasmid to form the relaxed molecule and subsequently changed to become a linear or open circular plasmid [58]. After the curing process, changes in the antibiotic resistance profile indicate a plasmid mediated, while unchanged profile indicated chromosomal mediated [36].

Even though studies on the prevalence and assessment of antibiotic resistance profile in Malaysia have been carried out, most were focused on *Vibrio parahaemolyticus, V. vulnificus, V. alginolyticus* and *V. cholerae* isolated from while leg shrimp, Asian seabass, tilapia and oyster, but not on grouper [23, 36, 44, 52]. Thus, this study aims to provide important information regarding prevalence, antibiotic resistance patterns and plasmid profiling of *Vibrio* spp. isolated from cultured groupers in Peninsular Malaysia.

Results

Clinical signs and gross lesions of groupers

A total of 150 (56%) of the 270 groupers were collected from nine farms were healthy and the remaining 120 (44%) were unhealthy due to observed clinical signs and gross lesions. Observations on the diseased groupers in all farms showed similar clinical abnormalities of vibriosis such as lethargy, loss of appetite and swimming on the surface of water. Based on 270 collected groupers, 107 (40%) had external and internal lesions suspecting of vibriosis, 57 (21%) had external lesions only, 29 (10%) had internal lesions only and 77 (29%) were asymptomatic.

The external lesions of vibriosis observed included ulcers on the skin, fins and mouth, corneal opacity, pop-eye and loss of one eyes. In advanced stage, affected fish showed discoloration or haemorrhagic skin (Fig. 1). Approximately 85% of the unhealthy and 30% of the healthy groupers had the external lesions. Upon dissection, examinations of the internal organs revealed 70% of the groupers had pale liver, 28% had soft and enlarged spleen, 14% with excessive ascetic fluids and less than 5% developed haemorrhagic liver and kidney with rotten organs (Fig. 2).

Prevalence of *Vibrio* spp.

A total of 380 suspected *Vibrio* strains were isolated based on the color of the colonies (green or yellow) that appeared on the thio-sulphate citrate bile salt sucrose

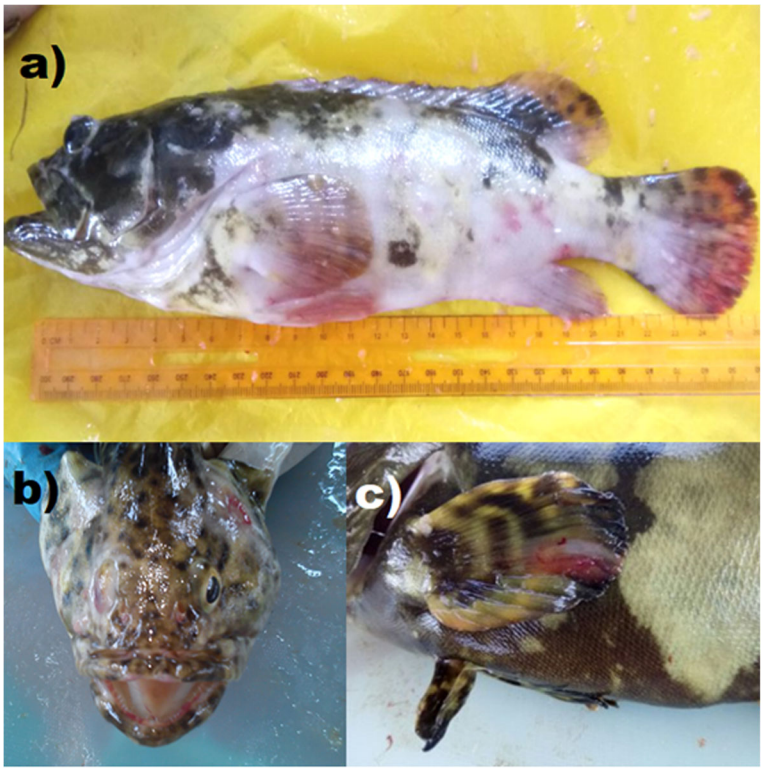

Fig. 1 External lesions of vibriosis observed on groupers including; **a** discoloration with lesion on the skin and fins, **b** loss on the left side of fish eye, **c** haemorrhagic on the pectoral fin

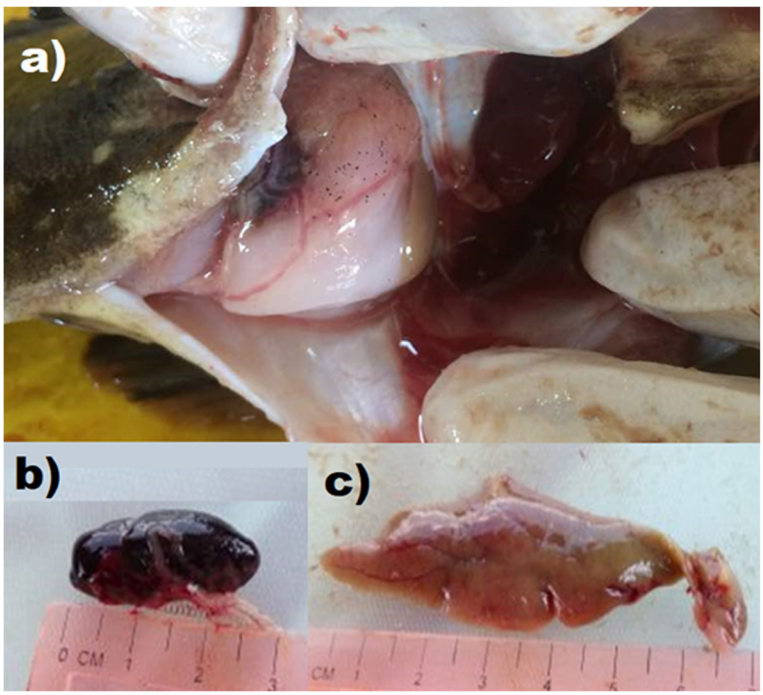

Fig. 2 Internal appearance of grouper infected by *Vibrio* spp. showing; **a** blood-tinged ascetic fluid, **b** enlargement of spleen (splenomegaly), **c** pale discolouration of liver

(TCBS) agar. They were isolated from 195 (72%) out of 270 groupers collected from nine farms in Peninsular Malaysia. Among them, 67 (18%) isolates were from Pulau Langkawi, Kedah, 66 (17%) from Pulau Ketam, Selangor, 58 (15%) from Kuala Gula, Perak, 54 (14%) from Port Dickson, Negeri Sembilan, 31 (8%) from Kota Bharu, Kelantan, 27 (7%) from Banting, Selangor, 27 (7%) from Kukup Laut, Johor, 26 (7%) from Jerteh, Terengganu, and 24 (6%) from Bukit Mertajam, Penang (Table 1).

All 380 isolates were Gram negative with biochemical characteristics of *Vibrio* spp. (Table 2). They were also *pyrH*-positive, producing the 440 bp band. Using phylogenetic analysis of the *pyrH* sequences, 13 *Vibrio* species were identified (Table 3, Fig. 3). The sequences reported have been deposited in the GenBank nucleotide sequence databases (accession numbers MN253135-MN253478) (See Additional file 1 for details).

The 380 *Vibrio* strains were successfully isolated and identified from 195 groupers. From the phylogenetic analysis, 110 (56%) groupers were infected with one species of *Vibrio,* 77 (39%) were infected with two different species of *Vibrio,* eight (4%) were infected with three different species of *Vibrio*. Of the 380 *Vibrio* strains, 106 (28%) were *V. communis,* 95 (25%) were *V. parahaemolyticus,* 70 (19%) were *V. alginolyticus,* 52 (14%) were *V. vulnificus,* 11(3%) were *V. rotiferianus* and *Vibrio* sp. and less than 3% were *V. campbellii, V. mytili, V. furnissii, V. harveyi, V. tubiashii, V. fluvialis* and *V. diabolicus.* Based on the sampling farms, *V. communis* was the most prominent isolated in Pulau Langkawi (67%), Jerteh (52%) and Bukit Mertajam (46%). *Vibrio parahaemolyticus* was dominant in Kota Bharu (61%) and Banting (56%). *Vibrio alginolyticus* was prominent in Pulau Ketam (52%), while in Kuala Gula farm, *V. parahaemolyticus* and *V. alginolyticus* were prominent at 38 and 36%„ respectively. *Vibrio Alginolyticus* (24%), *V. vulnificus* (20%) and *V. parahaemolyticus* (19%) were prominent in Port Dickson farm. In addition, *V. vulnificus* (44%) was also dominant in Kukup Laut.

Antimicrobial susceptibility profile

Antimicrobial susceptibility profile of the 380 *Vibrio* strains revealed 369 (97%) isolated were resistant to at least one antibiotic. Ninety-eight (27%) isolates were resistant to four antibiotics, followed by 82 (22%), 67 (18%), 66 (18%), 45 (12%), 10 (3%) and 1 (0.3%) isolates were resistant to 2, 5, 3, 1, 6 and 7 antibiotics, respectively. A total of 11 (3%) isolates were susceptible to all antibiotics tested.

A total of 303 (82%) *Vibrio* isolates were highly resistant to penicillin G and ampicillin, where 206 (56%), 166 (45%) and 115 (31%) isolates showed moderate resistant to vancomycin, bacitracin and erythromycin, respectively. Meanwhile, 54 (15%) isolates were resistant to tetracycline and 52 (14%) to streptomycin. Besides, 309

Table 1 The prevalence of *Vibrio* spp. isolated from groupers in each farm

State	Sampling area	No. of groupers infected with *Vibrio*	No. of *Vibrio* strains isolated	Organs	Species of *Vibrio* based on phylogenetic tree analysis
Kedah	Pulau Langkawi	29/30	67	Liver: 23, Spleen: 23, Kidney: 21	*V. communis* (45), *V. mytili* (7), *V. parahaemolyticus* (5), *V. vulnificus* (5), *V. rotiferianus* (3), *V. alginolyticus* (1), *Vibrio* sp. (1)
Penang	Bukit Mertajam	15/30	24	Liver: 2, Spleen: 14, Kidney: 8	*V. communis* (11), *V. vulnificus* (5), *V. tubiashii* (4), *V. harveyi* (2), *Vibrio* sp. (2)
Perak	Kuala Gula	25/30	58	Liver: 21, Spleen: 20, Kidney: 17	*V.parahaemolyticus* (22), *V. alginolyticus* (21), *V. campbellii* (6), *V. vulnificus* (5), *V. communis* (3), *V. rotiferianus* (1)
Kelantan	Kota Bharu	21/30	31	Liver: 13, Spleen: 9, Kidney: 9	*V.parahaemolyticus* (19), *V. campbellii* (2), *V. harveyi* (2), *V. mytili* (1), *V. alginolyticus* (1), *Vibrio* sp. (6)
Terengganu	Jerteh	17/30	26	Liver: 9, Spleen: 11, Kidney: 6	*V. communis* (14), *V. vulnificus* (11), *Vibrio* sp. (1)
Selangor	Pulau Ketam	24/30	66	Liver: 19, Spleen: 23, Kidney: 24	*V. alginolyticus* (34), *V.parahaemolyticus* (18), *V. communis* (12), *V. diabolicus* (1), *Vibrio* sp. (1)
	Banting	19/30	27	Liver: 11, Spleen: 8, Kidney: 8	*V.parahaemolyticus* (15), *V. communis* (9), *V. vulnificus* (3)
Negeri Sembilan	Port Dickson	28/30	54	Liver: 25, Spleen: 17, Kidney: 12	*V. alginolyticus* (13), *V. vulnificus* (11), *V.parahaemolyticus* (10), *V. furnissii* (7), *V. communis* (2), *V. fluvialis* (1), *V. campbellii* (1)
Johor	Kukup Laut	17/30	27	Liver: 6, Spleen: 16, Kidney: 5	*V. vulnificus* (12), *V. rotiferianus* (7), *V. parahaemolyticus* (6), *V. communis* (1), *V. harveyi* (1)
		195/270 (72%)	380	Liver: 129 (34%), Spleen: 141 (37%), Kidney: 110 (29%)	

Table 2 Identification of *Vibrio* spp. based on culture method, Gram stain and biochemical tests

Species	No. of isolates	TCBS	Gram stain	LDC	ONPG	Oxidase	Catalase	TSI
V. communis	106	Yellow	Gram negative	+	–	+	+	A/A, no gas, no H_2S
V. parahemolyticus	95	Green	Gram negative	+	–	+	+	A/A, no gas, no H_2S; K/A, no gas, no H_2S
V. alginolyticus	70	Yellow	Gram negative	+	–	+	+	A/A, no gas, no H_2S
V. vulnificus	52	Green	Gram negative	+	–	+	+	A/A, no gas, no H_2S
V. rotiferianus	11	Green	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. campbellii	9	Green	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. mytili	8	Green	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. furnissii	7	Yellow	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. harveyi	5	Yellow	Gram negative	+	–	+	+	A/A, no gas, no H_2S
V. tubiashii	4	Yellow	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. fluvialis	1	Green	Gram negative	–	–	+	+	A/A, no gas, no H_2S
V. diabolicus	1	Yellow	Gram negative	–	–	+	+	A/A, no gas, no H_2S
Vibrio sp.	11	Yellow	Gram negative	–	–	+	+	A/A, no gas, no H_2S

TCBS thio-sulphate citrate bile salt sucrose, *LDC* lysine decarboxylase, *ONPG* O-nitrophenyl-beta-D-galactosifase, *TSI* triple sugar iron, +: Positive, –: Negative, *A/A* Acidic slant/Acidic butt, *K/A* Alkaline slant/Acidic butt

(84%) isolates were highly susceptible to tetracycline, 248 (67%) isolates to streptomycin and 126 (34%) isolates to vancomycin (Fig. 4).

Antimicrobial susceptibility profile of the *Vibrio* spp.

The antimicrobial susceptibility profiles of the 13 identified species of *Vibrio* is summarised in Table 4. Most of the *Vibrio* spp. were highly resistant to ampicillin and penicillin G particularly *V. mytili* (100%), *V. tubiashii* (100%), *V. diabolicus* (100%), *V. fluvialis* (100%), *Vibrio* sp. (100%), *V. furnissii* (71–100%), *V. communis* (92–97%), *V. harveyi* (80%),*V. parahaemolyticus* (77–84%), *V. vulnificus* (64%), *V. alginolyticus* (61–79%) and *V. campbellii* (56%). Surprisingly, only 27% of the *V. rotiferianus* isolates were susceptible to ampicillin and penicillin G.

When tested with bacitracin, more than 50% *V. communis, V. vulnificus, V. tubiashii, V. fluvialis* and *V. campbellii* showed resistance pattern. The remaining eight *Vibrio* spp. showed intermediate and susceptible to bacitracin. In contrast, *V. rotiferianus* (91%) and *V. mytili* (88%) were highly resistant to erythromycin, while the remaining 11 *Vibrio* spp. showed intermediate and susceptible. High resistance of *Vibrio* spp. against vancomycin were observed among *V. fluvialis* (100%), *V. mytili* (88%), *V. communis* (70%), *V. rotiferianus* (64%), *V. parahaemolyticus* (57%), *V. furnissii* (57%) and *V. vulnificus* (54%). The other six *Vibrio* spp. (64–100%) showed intermediate and susceptible to vancomycin.

On the other hand, more than 80% isolates of nine *Vibrio* spp. were susceptible to tetracycline including *V. alginolyticus, V. campbellii, V. communis, V. diabolicus, V. harveyi, V. parahaemolyticus, V. rotiferianus, V. tubiashii* and *V. vulnificus.* In addition, 100% of *V. furnissii, V. fluvialis* and *V. diabolicus* were found susceptible to streptomycin, followed by 91% of *V. alginolyticus,* 89% of *V. campbellii,* 85% of *V. parahaemolyticus* and 73% of *V. rotiferianus.*

Plasmid profiles of *Vibrio* spp.

Among the 380 *Vibrio* isolates tested, 147 (39%) isolates harboured plasmid with molecular weight of more than 10 kb (Table 5) and 98 (67%) of them were resistant to four or more antibiotics. All *V. diabolicus,* 57% of *V. communis,* 46% of *V. rotiferianus,* 40% of *V. harveyi,* 37% of *V. vulnificus,* 34% of *V. parahaemolyticus,* 33% of *V. campbellii* and 29% of *V. alginolyticus* isolates were harboured plasmid. Meanwhile, less than 25% of *V. tubiashii*, *V. furnissii, V. mytili* and *Vibrio* sp. isolates harboured plasmid.

Following plasmid curing test, all isolates lost their plasmid DNA with 139 (95%) isolates showed altered resistance phenotype towards antibiotics. However, the isolates were remained resistant to either one or all antibiotics after plasmid curing, whereby 72% isolates remained resistance to ampicillin, 46% to penicillin G, 16% to bacitracin, 8% to vancomycin, 4% to erythromycin, 2%to tetracycline and 1% to streptomycin.

Multiplex antibiotic resistance (MAR) index

Overall, the mean MAR index value for *Vibrio* isolates was 0.44, with 85% showed MAR index value of more than 0.2. The most frequent MAR index for *Vibrio* spp. was 0.57, indicating that the isolates were resistance to four different antibiotics. In addition, the high MAR index value was observed among *V. fluvialis* (0.71), *V. tubiashii* (0.61), *V. communis* (0.57) and *V. mytili* (0.54). The other *Vibrio* spp. had MAR index value between 0.28 and 0.47, such as *V. furnissii* (0.47), *V. vulnificus* (0.45), *Vibrio* sp. (0.42), *V. harveyi* (0.4), *V. parahaemolyticus*

Table 3 List of reference sequences that related with *Vibrio* isolated from grouper in Malaysia

No	Species	No. of isolates	Reference sequences obtained from the GenBank database		
			Accession no	*Vibrio* spp.	Strain
1	*V. communis*	106	KC871657.1	*V. communis*	PEL26G
			KC871668.1	*V. communis*	PEL4D
			JX401895.1	*V. communis*	10G9
			GU078692.1	*V. communis*	R-40901
2	*V. parahemolyticus*	95	CP022243.1	*V. parahaemolyticus*	PB1937
			CP026041.1	*V. parahaemolyticus*	10,329
			CP014046.2	*V. parahaemolyticus*	ATCC 17802
			CP006004.1	*V. parahaemolyticus*	O1:Kuk
			CP003972.1	*V. parahaemolyticus*	BB22OP
			MG932062.1	*V. parahaemolyticus*	DSM 10027
3	*V. alginolyticus*	70	JN408273.1	*V. alginolyticus*	ATCC 17749
			CP014045.1	*V. alginolyticus*	FDAARGOS 114
			CP017919.1	*V. alginolyticus*	K09K1
			GU266285.1	*V. alginolyticus*	LMG 4409
4	*V. vulnificus*	52	CP019320.1	*V. vulnificus*	VV2014DJH
			CP012881.1	*V. vulnificus*	ATCC 27562
			CP012739.1	*V. vulnificus*	FORC_017
			CP014049.2	*V. vulnificus*	ATL6–1306
			CP009261.1	*V. vulnificus*	93 U204
5	*V. rotiferianus*	11	CP018312.1	*V. rotiferianus*	B64D1
			EF596722.1	*V. rotiferianus*	LMG21460
6	*V. campbellii*	9	EF596641.1	*V. campbellii*	LMG11216
			CP006605.1	*V. campbellii*	ATCC_BAA1116
			CP026315.1	*V. campbellii*	BoB-90
7	*V. mytili*	8	GU266287.1	*V. mytili*	LMG19157
8	*V. furnissii*	7	JF316672.1	*V. furnissii*	CAIM 518
9	*V. harveyi*	5	KC871684.1	*V. harveyi*	PEL36D
			CP025537.1	*V. harveyi*	ATCC 43516
10	*V. tubiashii*	4	LN998049.1	*V. tubiashii*	HLBLW2
			CP009345.1	*V. tubiashii*	ATCC19109
			GU186317.1	*V. tubiashii*	74 K
			MG932064.1	*V. tubiashii*	DSM19142
11	*V. fluvialis*	1	JN426808.1	*V.fluvialis*	LMG7894
12	*V. diabolicus*	1	CP014049.1	*V. diabolicus*	FDAARGOS 96
13	*Vibrio* sp.	11	EF394938.1	*Vibrio* sp.	RLUH-CZ
			JF739405.1	*Vibrio* sp.	CAIM 190

(0.4), *V. rotiferianus* (0.3), *V campbellii* (0.3), *V. diabolicus* (0.29) and *V. alginolyticus* (0.28).

Discussion

Grouper (*Epinephelus* spp.) has great commercial value worldwide including Malaysia due to high market price. Previous study reported that the production of grouper has increased, particularly in China, Indonesia, Philippines, Mexico and Pakistan [4]. However, high stocking density and poor handling of fish trigger disease outbreaks and mortality. In fact, two third of diseases reported in grouper was due to infection by *Vibrio* [12].

This study was successfully isolated 380 *Vibrio* bacteria from liver, spleen and/or kidney of 195 (72%)

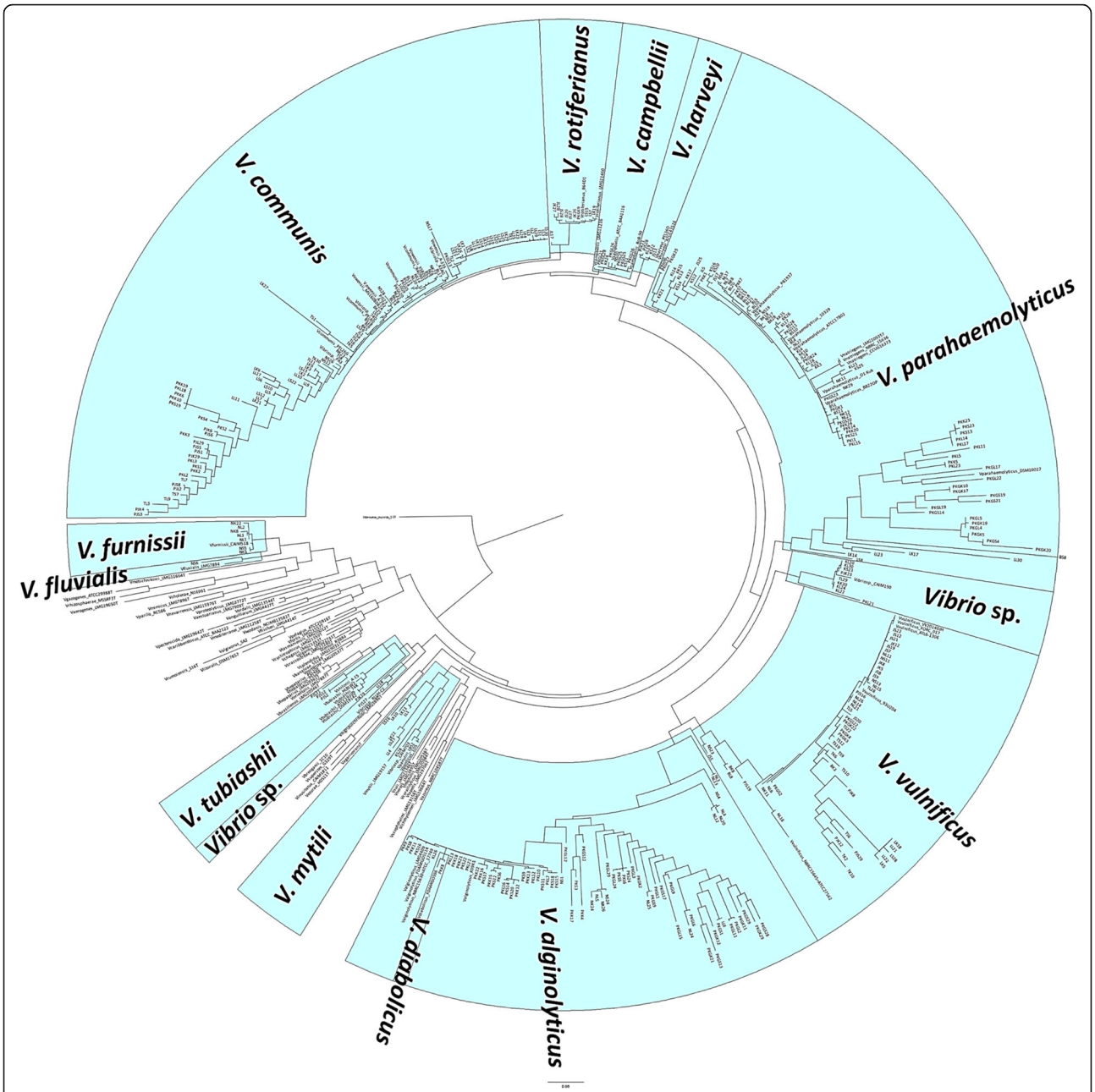

Fig. 3 Phylogenetic tree of *pyrH* gene sequences was inferred by using the ML method based on GTR + G model. Bootstrap values greater than 50% confidences were shown at branching points (percentages of 1000 replicates). The analysis involved 380 *Vibrio* isolate sequences and 102 reference sequences of *Vibrio* spp. (GenBank database). *P. damsela* subsp. *piscicida* strain D121 have been added as outgroup. Clusters highlighted in blue comprise the well-identified strains

groupers. The liver, spleen and kidney were known as vital organs for *Vibrio* infection [29]. In fact, Li et al. [38] had shown significant increased of *Vibrio* in spleen and kidney paralleled with the decline in macrophage phagocytosis of the infected fish. In addition, histology observation showed *Vibrio* was multiplied extensively in the kidney, liver and spleen of the infected fish [17].

The phylogenetic analysis of *pyrH* sequences revealed that 97% of the strains were clustered into 12 distinct species, with 3% strains were clustered into *Vibrio* sp. Among these 12 *Vibrio* species, *V. communis, V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* were highly isolated from groupers. It seemed that the *pyrH* gene could effectively distinguished the species level of

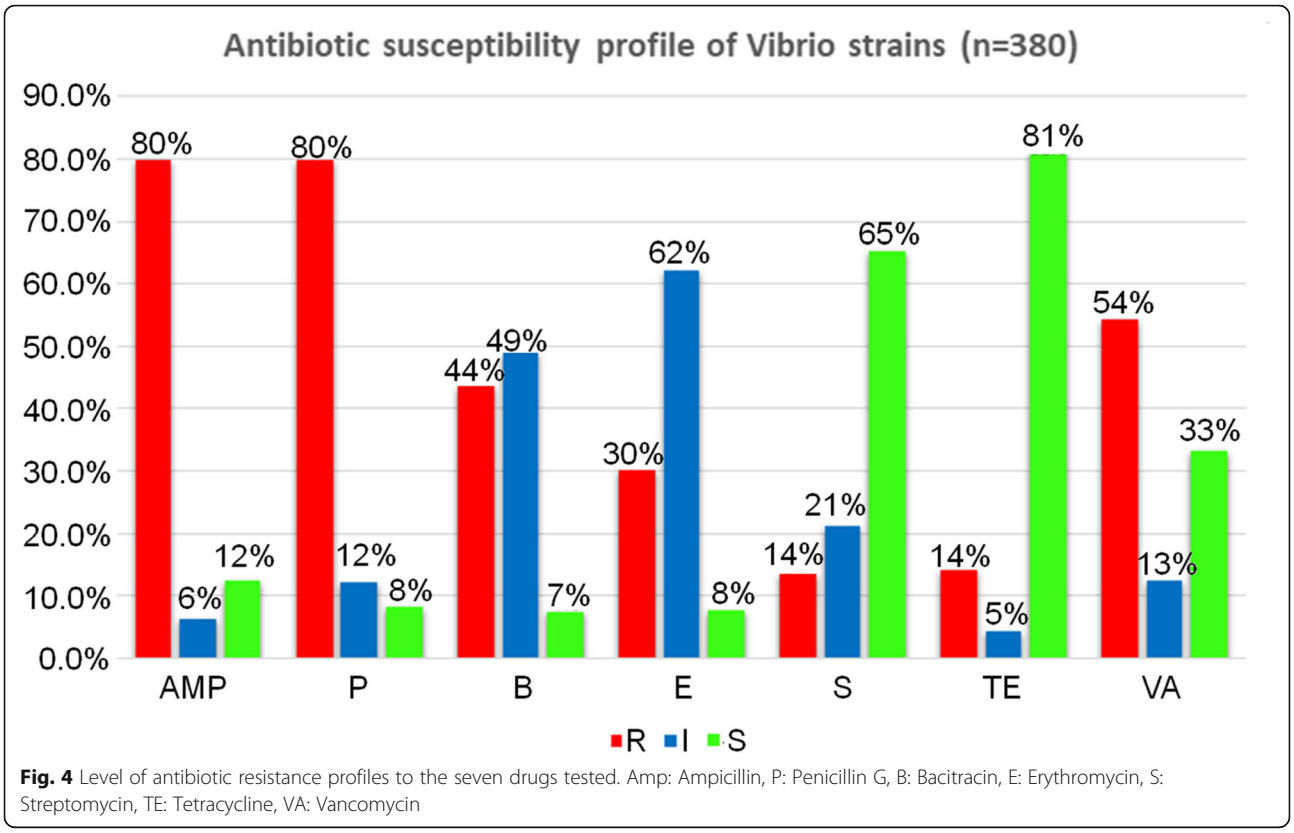

Fig. 4 Level of antibiotic resistance profiles to the seven drugs tested. Amp: Ampicillin, P: Penicillin G, B: Bacitracin, E: Erythromycin, S: Streptomycin, TE: Tetracycline, VA: Vancomycin

Vibrio including *V. communis*, which currently being described as *Vibrio* spp. [11]. Thus, the *pyrH* gene is a good phylo marker of *Vibrio* and a good discriminatory target at species level [48, 55, 59]. In addition, these findings are in agreement with previous studies that reported high presence of *V. alginolyticus*, *V. vulnificus* and *V. parahaemolyticus* within cultured tiger grouper (*Epinephelus fuscoguttatus*) in deep sea cage and other aquatic animals in Malaysian costal area [1, 19].

The antibiotic susceptibility test found that they were resistant to ampicillin, penicillin G and vancomycin, highly susceptible to tetracycline and streptomycin and intermediate against bacitracin and erythromycin. In fact, 64% of the *Vibrio* isolates were resistance to at least

Table 4 Antibiotic resistance profiles based on the *Vibrio* spp.

Vibrio species	No of isolates	AMP			P			B			E			S			TE			VA		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
V. communis	106	100	5	1	103	2	1	60	43	3	49	49	8	25	46	35	16	1	89	74	14	18
V. parahaemolyticus	95	73	7	15	80	7	8	36	53	6	18	71	6	5	9	81	8	6	81	54	6	35
V. alginolyticus	70	55	7	8	43	19	8	17	48	5	6	59	5	1	5	64	9	3	58	25	4	41
V. vulnificus	52	33	8	11	33	13	6	29	19	4	20	28	4	15	7	30	13	3	36	28	12	12
V. rotiferianus	11	3	0	8	3	4	4	4	2	5	10	0	1	0	3	8	1	0	10	7	1	3
V. campbellii	9	5	0	4	5	0	4	6	3	0	2	7	0	0	1	8	1	0	8	2	1	6
V. mytili	8	8	0	0	8	0	0	0	8	0	7	1	0	0	7	1	0	3	5	7	1	0
V. furnissii	7	5	2	0	7	0	0	3	1	3	0	5	2	0	0	7	5	1	1	4	2	1
V. harveyi	5	4	1	0	4	1	0	2	2	1	1	3	1	2	0	3	0	0	5	1	2	2
V. tubiashii	4	4	0	0	4	0	0	4	0	0	1	3	0	2	0	2	0	0	4	0	0	4
V. diabolicus	1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0	1
V. fluvialis	1	1	0	0	1	0	0	1	0	0	0	0	1	0	0	1	1	0	0	1	0	0
Other *Vibrio* sp.	11	11	0	0	11	0	0	4	6	1	1	9	1	3	2	6	0	0	11	3	5	3

R resistance, *I* intermediate, *S* susceptibility, *AMP* ampicillin, *P* penicillin G, *B* bacitracin, *E* erythromycin, *S* streptomycin, *TE* tetracycline, *VA* vancomycin

Table 5 The antibiotic resistance profile patterns of *Vibrio* spp. before and after plasmid curing

Species	Strain number	Before plasmid curing Antibiotic profiles	No of plasmid	After plasmid curing Antibiotic profiles	No of plasmid
V. alginolyticus	PKGK2	Amp/ E/ VA	1	B	Lost
V. alginolyticus	PKGL9, PKGL12, PKS18	Amp/ P	1	Amp	Lost
V. alginolyticus	PKS3	Amp/ P	1	Amp/ P	Lost
V. alginolyticus	PKL7, PKL13	Amp/ P	1	No resistance	Lost
V. alginolyticus	PKGS1, PKS12	Amp/ P/ VA	1	Amp	Lost
V. alginolyticus	PKGK21	Amp/ P/ VA	1	Amp/ VA	Lost
V. alginolyticus	PKS15	Amp/ P/ VA	1	No resistance	Lost
V. alginolyticus	NL3	Amp/ P/ B	1	Amp/ P/ B	Lost
V. alginolyticus	PKGL1	Amp/ P/ B/ E	1	Amp/ P	Lost
V. alginolyticus	LL6	Amp/ P/ B/ E/ VA	1	Amp/ P	Lost
V. alginolyticus	NS4	Amp/ P/ B/ TE/ VA	1	VA	Lost
V. alginolyticus	NL5	Amp/ P/ B/ TE/ VA	1	Amp/ P/ B	Lost
V. alginolyticus	PKGL15	Amp/ P/ B/ VA	1	No resistance	Lost
V. alginolyticus	NK26	Amp/ P/ B/ VA	1	Amp/ P/ B	Lost
V. alginolyticus	PKGS29	E/ VA	1	E/ VA	Lost
V. alginolyticus	PKK15	P/ VA	1	No resistance	Lost
V. campbellii	PKGL21	Amp/ P	1	Amp	Lost
V. campbellii	PKGL28	Amp/ P/ B	1	B	Lost
V. campbellii	NS30	Amp/ P/ B/ TE/ VA	1	Amp/ P/ B	Lost
V. communis	PKS1, PKL18, PKGL20	Amp/ P	1	Amp	Lost
V. communis	LS22	Amp/ P/ B/ E/ VA	1	No resistance	Lost
V. communis	LK27	Amp/ P/ B/ E/ S/ TE/ VA	1	Amp/ TE	Lost
V. communis	LL7, LS10, LS12	Amp/ P/ B/ E/ S/ VA	1	Amp/ P	Lost
V. communis	LL9, LK1	Amp/ P/ B/ E/ TE/ VA	1	Amp/ P	Lost
V. communis	LS21	Amp/ P/ B/ E/ TE/ VA	1	Amp/ P/ TE	Lost
V. communis	TL11, LK4, LK16	Amp/ P/ B/ E/ VA	1	Amp	Lost
V. communis	LK3, LS6, LK6, LK9, LL10, LL17, LS1, LS3, LL13, LK13, LL19, LL29, LK29	Amp/ P/ B/ E/ VA	1	Amp/ P	Lost
V. communis	LS13	Amp/ P/ B/ E/ VA	1	No resistance	Lost
V. communis	PJL2, PJS5	Amp/ P/ B/ S	1	Amp/ P/ S	Lost
V. communis	PJK4	Amp/ P/ B/ S	1	Amp/ P/ B	Lost
V. communis	PJS8, PJL29	Amp/ P/ B/ S	1	Amp/ P	Lost
V. communis	NS12	Amp/ P/ B/ TE	1	P/ B	Lost
V. communis	NS14	Amp/ P/ B/ TE	1	No resistance	Lost
V. communis	NL30	Amp/ P/ B/ TE/ VA	1	No resistance	Lost
V. communis	NS28	Amp/ P/ B/ TE/ VA	1	Amp/ P/ B	Lost
V. communis	TL3	Amp/ P/ B/ VA	1	Amp	Lost
V. communis	PKGL13	Amp/ P/ B/ VA	1	Amp/ P/ B	Lost
V. communis	PKL3; PKK3	Amp/ P/ E/ S	1	Amp	Lost
V. communis	LK21, LL15	Amp/ P/ E/ S/ VA	1	Amp	Lost
V. communis	LL5, LS17, LS25	Amp/ P/ E/ VA	1	Amp	Lost
V. communis	LL3, LL8, LS19, LL28	Amp/ P/ E/ VA	1	Amp/ P	Lost
V. communis	PKS2	Amp/ P/ S	1	Amp	Lost

Table 5 The antibiotic resistance profile patterns of *Vibrio* spp. before and after plasmid curing *(Continued)*

Species	Strain number	Before plasmid curing	No of plasmid	After plasmid curing	No of plasmid
		Antibiotic profiles		Antibiotic profiles	
V. communis	BS2	Amp/ P/ VA	1	Amp/ VA	Lost
V. communis	PKS4, BL17	Amp/ P/ VA	1	Amp	Lost
V. communis	BS18	Amp/ P/ VA	1	Amp/ P	Lost
V. communis	PKL2, PKK6, PKS19, BS14	Amp/ P/ VA	1	No resistance	Lost
V. diabolicus	PKK8	Amp/ P	1	No resistance	Lost
V. furnissii	NL2	Amp/ P/ TE/ VA	1	No resistance	Lost
V. harveyi	PJK21, PJS28	Amp/ P/ B/ S	1	Amp/ P	Lost
V. mytili	LS23	Amp/ P/ E/ VA	1	Amp	Lost
V. parahaemolyticus	PKL11	Amp/ P	1	No resistance	Lost
V. parahaemolyticus	PKGS23	Amp/ P/ VA	1	VA	Lost
V. parahaemolyticus	PKGS11	Amp/ P/ B/ E	1	Amp/ P/ B/ E	Lost
V. parahaemolyticus	LL23	Amp/ P/ B/ E/ VA	1	Amp	Lost
V. parahaemolyticus	LL30	Amp/ P/ B/ E/ VA	1	Amp/ P	Lost
V. parahaemolyticus	PKGS6	Amp/ P/ B/ E/ VA	1	Amp/ P/ B/ E	Lost
V. parahaemolyticus	PKGL19	Amp/ P/ B/ E/ VA	1	Amp/ B/ VA	Lost
V. parahaemolyticus	PKGK24	Amp/ P/ B/ E/ VA	1	No resistance	Lost
V. parahaemolyticus	NK6	Amp/ P/ B/ TE	1	Amp/ P/ B	Lost
V. parahaemolyticus	NS27	Amp/ P/ B/ TE/ VA	1	P/ B/ VA	Lost
V. parahaemolyticus	PKGL17	Amp/ P/ B/ VA	1	Amp/ P/ B/ VA	Lost
V. parahaemolyticus	PKGL22	Amp/ P/ B/ VA	1	Amp/ B/ VA	Lost
V. parahaemolyticus	BL4, BS11, BK12, BL21	Amp/ P/ B/ VA	1	Amp/ P	Lost
V. parahaemolyticus	PKS27, BS28, BK28	Amp/ P/ B/ VA	1	Amp	Lost
V. parahaemolyticus	PKK14	Amp/ P/ B/ VA	1	No resistance	Lost
V. parahaemolyticus	PKGK19	Amp/ P/ E	1	Amp/ P	Lost
V. parahaemolyticus	NK29	Amp/ P/ TE	1	Amp/ P	Lost
V. parahaemolyticus	PKK7, BS8	Amp/ P/ VA	1	Amp	Lost
V. parahaemolyticus	PKL14	Amp/ P/ VA	1	No resistance	Lost
V. parahaemolyticus	PKL17	Amp/ P/ VA	1	Amp/ P	Lost
V. parahaemolyticus	PKK24	Amp/ P/ VA	1	P	Lost
V. parahaemolyticus	PKGK17	E/ VA	1	E	Lost
V. parahaemolyticus	PKGL4	P/ B/ VA	1	P/ B/ VA	Lost
V. parahaemolyticus	PKGL5, PKL1	P/ B/ VA	1	No resistance	Lost
V. parahaemolyticus	PKK23	P/ S	1	No resistance	Lost
V. rotiferianus	LS7	Amp/ P/ B/ E/ VA	1	Amp/ P	Lost
V. rotiferianus	LS15	Amp/ P/ E/ VA	1	Amp/ P	Lost
V. rotiferianus	LK19	Amp/ P/ B/ E/ VA	1	Amp	Lost
V. rotiferianus	PKGK9	B/ E/ VA	1	No resistance	Lost
V. rotiferianus	JS28	B/ E/ TE/ VA	1	TE/ VA	Lost
V. tubiashii	PJK19	Amp/ P/ B/ S	1	Amp/ P/ B	Lost
V. vulnificus	LK5, LS28	Amp/ P/ E/ VA	1	Amp	Lost
V. vulnificus	LL21	Amp/ P/ E/ S/ VA	1	Amp	Lost
V. vulnificus	PJK12	Amp/ P/ B	1	Amp/ P/ B	Lost
V. vulnificus	LK18, TS22	Amp/ P/ B/ E/ S/ VA	1	Amp/ P	Lost

Table 5 The antibiotic resistance profile patterns of *Vibrio* spp. before and after plasmid curing *(Continued)*

Species	Strain number	Before plasmid curing Antibiotic profiles	No of plasmid	After plasmid curing Antibiotic profiles	No of plasmid
V. vulnificus	TL5, TS6	Amp/ P/ B/ E/ S/ VA	1	No resistance	Lost
V. vulnificus	PJK8	Amp/ P/ B/ S	1	Amp/ P/ B	Lost
V. vulnificus	PJS16	Amp/ P/ B/ S	1	Amp/ P	Lost
V. vulnificus	NL15	Amp/ P/ B/ TE/ VA	1	Amp/ P/ B	Lost
V. vulnificus	TS19	Amp/ P/ B/ VA	1	Amp	Lost
V. vulnificus	TK2	Amp/ P/ E/ VA	1	Amp	Lost
V. vulnificus	PKGK3	Amp/ P/ VA	1	Amp/ P/ VA	Lost
V. vulnificus	BL8	Amp/ P/ VA	1	Amp	Lost
V. vulnificus	JS9	B/ E/ S/ TE/ VA	1	B/ E	Lost
V. vulnificus	JS19	E/ S/ TE/ VA	1	E/ VA	Lost
V. vulnificus	PJS19	P/ B	1	P	Lost
V. vulnificus	BK3	P/ S	1	No resistance	Lost
Vibrio spp.	LL18	Amp/ P/ B/ E/ VA	1	Amp/ P	Lost
Vibrio spp.	PKL21	Amp/ P/ B/ S/ VA	1	No resistance	Lost

AMP ampicillin, *P* penicillin G, *B* bacitracin, *E* erythromycin, *S* streptomycin, *TE* tetracycline, *VA* vancomycin

three or more antibiotics. These findings were similar with a previous study reported that 68% of the *Vibrio* isolates were resistance to at least three or more antibiotics [65].

Based on MAR index, the isolates have been continuously exposed to antibiotics since the mean value calculated among 380 isolates was 0.44 [23]. There were also 85% isolates having MAR index value of more than 0.2, which indicate high risk of contamination with potentially hazardous to human health [62]. This is in agreement with previous studies done in Malaysian aquaculture [44, 52, 68].

The antibiotic susceptibility profiles obtained in current study clearly indicate that tetracycline and streptomycin remained highly effective against *Vibrio* spp., including *V. communis, V. parahaemolyticus, V. alginolyticus, V. vulnificus* and *V. rotiferianus.* This was supported by previous studies on the effectiveness of both antibiotics for the treatment against *Vibrio* spp. in Malaysia [24, 44]. In addition, many studies have proven that *V. parahaemolyticus* isolated from fish and other aquatic animals was susceptible to tetracycline and streptomycin [24, 32, 35, 44, 46, 47, 61, 66].

This study also found that 70 and 56% of *Vibrio* isolates were intermediate and susceptible against erythromycin and bacitracin, respectively, while 30% of *Vibrio* isolates mainly *V. rotiferianus* and *V. mytili* were highly resistance against erythromycin. According to Kumar et al. [32], *Vibrio* spp. isolated from seafood samples from coastal India were resistant to ampicillin, penicillin and erythromycin, while 44% of the *Vibrio* isolates were found resistance to bacitracin. This is slightly less compared to 98% in a study by Sahilah et al. [51] who studied the resistance to bacitracin among *V. parahaemolyticus* in cockle. The discrepancies regarding the resistance of *Vibrio* to antibiotic could possibly be due to geographical variation or difference in test methodology [36].

In this study, *Vibrio* spp. showed high resistance toward ampicillin and penicillin G. Previous reports showed resistance of both antibiotics in *Vibrio* are not a new phenomenon. Zanetti et al. [67] reported that *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* isolated from seawater were highly resistance to ampicillin. Another study reported that 81% of *V. parahaemolyticus* isolated from oyster were resistance to ampicillin [24]. Similarly, *V. parahaemolyticus* isolated from croaker fish (*P. senegalensis*) and blue crab (*Callinectes sapidus*) at Lagos Lagoon, Nigeria, showed resistance to ampicillin [46]. In China, 79.6% of *V. parahaemolyticus* isolated from fish, shrimp and oyster were resistant to ampicillin [65].

In addition, Vaseeharan et al. [64] reported the emergence of resistant *Vibrio* strains against ampicillin and penicillin in India. Over 80% of *V. harveyi* from fish in Italy showed resistant to ampicillin, amoxicillin and erythromycin [56]. The findings were also in agreement with studies done all around world and Malaysia [2, 5, 18, 52, 61]. Emergence of high resistance *Vibrio* strains against ampicillin and penicillin was related with the extensive used of both antibiotics and could influence the disease management in aquaculture system [21]. Thus, both ampicillin and penicillin are ineffective for treatment of *Vibrio* infection [61]. Instead of ampicillin and penicillin, seven out of 13

Vibrio spp. were found to be highly resistant against vancomycin. This finding was consistent with a previous study in Selangor, Malaysia, that reported by Noorlis et al. [44]. In addition, a study done in South Korea showed that all *V. parahaemolyticus* isolated from oysters were resistance to ampicillin and vancomycin [30].

The plasmid profiling revealed low occurrence of plasmid (39%), indicating that the resistance genes were of chromosomal mediated. Manjusha and Sarita [41] also revealed that 21 (70%) out of 30 *Vibrio* isolates did not exhibit plasmids but still resistance to all antibiotics. In addition, previous studies on *Vibrio* found no correlation between resistance to the antibiotics and the presence of plasmid [16, 67]. On the other hand, this study found that 67% isolates with plasmid were resistance to more than four antibiotics, indicating that the presence of plasmids might enhanced the virulence and antibiotic resistance [16, 49].

This study also revealed that the resistance to all antibiotics especially to ampicillin, penicillin G, bacitracin and vancomycin was related to the chromosome since the isolates remained resistant to these antibiotics after plasmid curing. Similar results were demonstrated in other studies by Reboucas et al. [50] and Costa et al. [14]. A study reported that the β-lactamase involved in ampicillin resistance was found to be chromosomally encoded in *V. harveyi* [28]. Thus, the antibiotic resistance genes in *Vibrio* spp. isolated from grouper were found in both plasmid and chromosome.

Conclusion

In conclusion, our findings represent a comprehensive report on the antibiotic resistance profiles and plasmid curing of *Vibrio* spp. isolated from groupers in Malaysia. The vancomycin, bacitracin and erythromycin resistance patterns suggested that treatment of vibriosis with these antibiotics need to be reconsidered. By reducing the usage of these antibiotics may consequence the decrease in antibiotic resistance. Hence, continuous monitoring of susceptibility of *Vibrio* strains to antibiotics is necessary to ensure the best treatment and combat drug resistance among them.

Methods

Sampling of groupers

A total of 210 hybrid grouper (*Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂) and 60 green groupers (*E. suillus*) were obtained from nine farms that were located in different geographical regions of Peninsular Malaysia (Table 6). Fish were obtained during the period between December 2016 and September 2017. Thirty fish were randomly collected from each farm and the size of fish varies ranging between 14 and 580 g in weight, and between 10 and 31 cm in length. Any clinical signs and gross lesions of vibriosis were observed and documented.

Euthanasia and dissection of fish was performed at the sampling sites. Fish was euthanized in 0.2% of tricaine methanesulfonate (Western Chemical Industries, Mumbai, India). Fish was dissected for the collection of liver, kidney and spleen. These organs were immersed separately in 1× phosphate buffered saline (PBS) (Merck, New Jersey, USA). The samples were kept in ice and transported to the laboratory for processing on the same day.

Isolation of *Vibrio* from liver, spleen and kidney

The liver, spleen and kidney of the groupers were separately homogenized using stomacher for 1 min. The homogenized sample was then streaked on thio-sulphate citrate bile salt sucrose (TCBS) agar (Difco, Michigan, USA) and incubated at 30 °C for 16 h. A single colony of bacteria suspected of *Vibrio* was incubated in tryptic soy broth (TSB) (Difco) with 1.5% normal saline (Merck) and incubated at 30 °C for 16 h. Alternate steps between TCBS and TSB containing 1.5% normal saline were performed until a pure colony of *Vibrio* was obtained. A pure isolate was inoculated into semi-solid nutrient agar and TSB with 20% glycerol, incubated at 30 °C for 16 h and then stored until further analysis.

Table 6 List of farms

	Farm	Location	GPS Location
1	Widad Agrofarm Sdn. Bhd.	Pulau Langkawi, Kedah	6°14′38.04″N, 99°57′11.88″E
2	Weng Teik Shrimp Farm	Bukit Mertajam, Penang	5°20′33.972″N, 100°26′36.96″E
3	Ain Aquaculture Sdn. Bhd.	Kota Bharu, Kelantan	6°7′59.808″N, 102°14′18.96″E
4	Perniagaan Johari	Besut, Terengganu	5°34′15.852″N, 102°31′8.795″E
5	Aqua Hub Sdn. Bhd.	Kuala Gula, Perak	4°59′51.203″N, 100°24′18.032″E
6	KS Aquaculture Sdn. Bhd.	Pulau Ketam, Selangor	3°2′9.348″N, 101°14′34.799″E
7	Oasis Long Diann Bio-Tech Sdn. Bhd.	Banting, Selangor	2°49′12.216″N, 101°30′56.404″E
8	Aqua Genesis Sdn. Bhd.	Port Dickson, Negeri Sembilan	2°32′13.848″N, 101°48′21.6″E
9	Smart Objectives Sdn. Bhd.	Kukup Laut, Johor	1°25′22.8″N, 103°26′32.999″E

Identification of *Vibrio* spp. using gram stain, biochemical tests, *pyrH*-PCR assay and sequencing

All pure colonies were subjected to Gram staining (Becton Dickinson, New Jersey, USA) and biochemical tests (triple sugar iron (TSI), oxidase, catalase, O-nitrophenyl-beta-D-galactosifase (ONPG) and lysine decarboxylase (LDC) (Oxoid, Hampshire, UK) for identification of the *Vibrio* spp. [7, 27].

Genomic DNA of pure colonies were extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's protocol. The genomic DNA was subjected to PCR amplification using *pyrH* primers; *pyrH*_F (5′-GAT CGT ATG GCT CAA GAA G-3′) and *pyrH*_R (5′-TAG GCA TTT TGT GGT CAC G-3′) [10]. The PCR reactions were performed in a final volume of 50 μL containing 1× PCR buffer, 3 mM $MgCl_2$, 200 uM dNTPs, 0.5 pmol of each primer, 2.5 U *Taq* polymerase and 50 ng of template DNA (Promega, Wisconsin, USA). The *pyrH* cycle condition was an initial denaturation at 95 °C for 5 s, followed by 33 cycles of 95 °C for 1 min; 59 °C for 2 min 15 s and 72 °C for 1 min 15 s, and a final extension of 72 °C for 10 s. The amplification was performed in an Eppendorf Mastercycler Nexus Thermal Cycler (Eppendorf, Hamburg, Germany).

Direct sequencing of purified PCR products was performed on sense strands (First Base, Kuala Lumpur, Malaysia). Phylogenetic analysis was conducted using MEGA version 7.0 [33]. The phylogenetic construction of *pyrH* genes of *Vibrio* isolates and reference sequences (obtained from GenBank database) was inferred using the Maximum Likelihood (ML) method based on the General Time Reversible (GTR) model and 1000 rapid bootstrap inferences [43].

Antibiotic susceptibility test

The *Vibrio* isolates were assessed for their antibiotic susceptibility by disc diffusion method as described by Devi et al. [16]. Seven antibiotics (Thermo Fisher Scientific, Massachusetts, USA) were used, which included tetracycline 30 μg (TE), ampicillin 10 μg (AMP), penicillin G 10 μg (P), streptomycin 10 μg (S), erythromycin 15 μg (E), vancomycin 30 μg (VA) and bacitracin 10 μg (B).

Vibrio suspension of approximately 1×10^8 CFU/mL was inoculated by lawn on Muller-Hinton agar (MHA) (Difco) using a cotton swab. The antibiotic discs were then placed 15 mm away from the edge of the plates to prevent overlapping of the zones of inhibition. After incubation at 37 °C for 24 h, the diameter of inhibition zone was measured. Strain was then regarded as resistance, intermediate or susceptible based on guidelines of the Clinical and Laboratory Standards Institute (CLSI) [13].

Plasmid profiling

A total of 2.5 mL of bacterial culture from TSB supplemented with 1.5% normal saline was centrifuged at 12000×g for 3 min. A *Vibrio* isolate was purified using GeneJet Plasmid Purification kit according to the manufacturer's protocol (Thermo Fisher Scientific)). The supernatant containing plasmid was kept at -20 °C until used. Presence of plasmid was detected using the agarose gel (1% w/v) electrophoresis (Bio-Rad Laboratories, California, USA).

Plasmid curing

Vibrio isolates that harboured plasmid were treated with the acridine orange (AO) (Thermo Fisher Scientific) following modifications of the methods by Letchumanan et al. [37]. A single colony of bacterial isolate from TCBS agar was grown on Tryptone Soy Broth (TSB) supplemented with 1.5% NaCl and 0.2 mg/mL AO. Bacterial culture was incubated at 37 °C for 24 h under constant agitation. After treatment with curing agent, the agarose gel (1% w/v) electrophoresis was performed to detect for the presence of plasmid. In addition, to verify changes in resistance profiles, the antibiotic susceptibility test was again performed as described previously.

Multiplex antibiotic resistance index

Multiplex antibiotic resistance (MAR) index was calculated based on the ratio of resistance antibiotics to the total number of antibiotics to which the isolates are exposed to [31]. The MAR index provides an accurate estimation about the origin of contamination [18].

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-019-1624-2.

Additional file 1. The sequences of oligonucleotide used in this study.

Abbreviations

A/A: Acidic slant/Acidic butt; Amp: Ampicillin; AO: Acridine orange; B: Bacitracin; E: Erythromycin; EtBr: Ethidium bromide; GTR: General Time Reversible; K/A: Alkaline slant/Acidic butt; LDC: Lysine decarboxylase; MAR: Multiple antibiotic resistance; MHA: Muller-Hinton agar; ML: Maximum Likelihood; ONPG: O-nitrophenyl-beta-D-galactosidase; P: Penicillin G; PBS: Phosphate buffered saline; S: Streptomycin; SDS: Sodium dodecyl sulphate; Spp: Species; TCBS: Thiosulphate citrate bile salt sucrose; TE: Tetracycline; TSB: Tryptone soy broth; TSI: Triple sugar iron; VA: Vancomycin

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Authors' contributions

DZ, MNAA, MTY, MZ and IMY participated in design the experiment and helped supervise the project. NAZ and SS carried out all the experiment. The manuscript was written by NAZ. All authors provided critical feedback, helped shape the research, analysis and approved the final manuscript.

Authors' information
The selected sequences that represent *Vibrio* species in each farm have been deposited in the GenBank nucleotide sequence databases (accession numbers MN253135-MN253478). The selected sequences generated or analysed during this study are included in this published article [Additional file 1].

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
In this study, experiment was not performed on live vertebrates. Instead, freshly caught dead fish was used and therefore no ethics approval is required. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM) under application number UPM/IACUC/AUP/R059/2016. Permission were obtained from the farm-owners (Personal communication) in order to collect the fish using non-lethal methods.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Abdullah, A., Nur-Nazifah, M., Rimatulhana, N. R., Syafiq, M. S., Abdullah, M. Z. and Siti-Zahrah, A. Concurrent infections in tiger grouper (*Epinephelus fuscoguttatus*) cultured in deep sea cages in Langkawi. 2015. http://irep.iium.edu.my/47308/. Accessed 12 Oct 2018.
2. Al-Othrubi SMY, Kqueen CY, Mirhosseini H, Hadi YA, Radu S. Antibiotic resistance of *Vibrio parahaemolyticus* isolated from cockles and Shrimp Sea food marketed in Selangor, Malaysia. Clin Microbiol. 2014;3:148.
3. Amal MNA, Zamri-Saad M, Iftikhar AR, Siti-Zahrah A, Aziel S, Fahmi S. An outbreak of Streptococcus agalactiae infection in cage-cultured golden pompano, Trachinotus blochii (Lacépède, 1801), in Malaysia. J Fish Dis. 2012; 35:849–52.
4. Amorim P, Westmeyer M. Snapper and Grouper: SFP Fisheries Sustainability Overview 2015: Sustainable Fisheries Partnership Foundation; 2016. p. 18. Available from http://www.fishsource.com.
5. Ang GY, Yu CY, Balqis K, Elina HT, Azura H, Hani MH, Yean CY. Molecular evidence of cholera outbreak caused by a toxigenic *Vibrio cholerae O1 El Tor* variant strain in Kelantan, Malaysia. J Clin Microbiol. 2010;48(11):3963–9.
6. Bondad-Reantaso MG. Disease and health management in Asian aquaculture. Vet Parasitol. 2005;132(3–4):249–72.
7. Buller NB. Bacteria and Fungi from fish and other aquatic animals: a practice identification manual. 2nd ed. Wallingford: CABI Publishing; 2015.
8. Cabello FC. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol. 2006;8(7):1137–44.
9. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Doiz H, Millanao A, Buschmann AH. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol. 2013;15(7):1917–42.
10. Chimetto LA, Brocchi M, Gondo M, Thompson CC, Gomez-Gil B, Thompson FL. Genomic diversity of vibrios associated with the Brazilian coral Mussismilia hispida and its sympatric zoanthids (*Palythoa caribaeorum, Palythoa variabilis* and *Zoanthus solanderi*). J Appl Microbiol. 2009;106:1818–26.
11. Chimetto LA, Cleenwerck I, Alves NJ, Silva BS, Brocchi M, Willems A, De VP, Thompson FL. *Vibrio communis sp. nov.*, isolated from the marine animals *Mussismilia hispida, Phyllogorgia dilatata, Palythoa caribaeorum, Palythoa variabilis* and *Litopenaeus vannamei*. Int J Syst Evol Microbiol. 2011;61(2):362–8.
12. Chong, R., Bousfield, B. and Ricard, B. Fish disease management. Veterinary Bulltetin-Agriculture, Fisheries and Conservative Department Newsletter 2011;1(8):1–12.
13. CLSI. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious Bacteria; approved guideline. 2nd ed. Clinical and Laboratory Standards Institute: Wayne; 2010. CLSI document M45-A2
14. Costa AR, Araujo RL, Souza OV, Vieira RH. Antibiotic-resistance vibrios in farmed shrimp. Biomed Res Int. 2015;505914:1–5.
15. Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol. 2007;25(10):472–9.
16. Devi R, Surendran PK, Chakraboorty K. Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from shrimp farms along the southwest coast of India. World J Microbiol Biotechnol. 2009;25:2005–12.
17. Diggles BK, Carson J, Hine M, Hickman RW, Tait MJ. *Vibrio* species associated with mortalities in hatchery-reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in New Zealand. Aquaculture. 2000;183:1–12.
18. Elexson N, Afsah-Hejri L, Rukayadi Y, Soopna P, Lee HY, Zainazor TCT, Ainy NM, Nakaguchi Y, Mitsuaki N, Son R. Effect of detergents as antibacterial agents on biofilm of antibiotics-resistance *Vibrio parahaemolyticus* isolates. Food Control. 2014;35(1):378–85.
19. Elhadi N, Radu S, Chen CH, Nishibuchi M. Prevalence of potentially pathogenic *Vibrio* species in the seafood marketed in Malaysia. J Food Prot. 2004;67(7):1469–75.
20. Elmahdi S, DaSilva LV, Parveen S. Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. Food Microbiol. 2016;57:128–34.
21. FAO. Antimicrobial Resistance (AMR) in aquaculture. Rome; 2017. Available from http://www.fao.org/cofi/aq/90408/en/.
22. FAO. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome; 2018. Licence: CC BY-NC-SA 3.0 IGO.
23. Hamdan RH, Peng TL, Ong BL, Suhana MYS, Hamid NH, Afifah MNF, Raina MS. Antibiotics Resistance of *Vibrio* spp. Isolated from Diseased Seabass and Tilapia in Cage Culture. Proc. Intsem. LPVT. 2016;2016:554–60.
24. Han F, Walker RD, Janes ME, Prinyawiwatkul W, Ge B. Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana gulf and retail raw oysters. Appl Environ Microbiol. 2007; 73:7096–8.
25. Ibrahim AB, Khan MA, Ayob MY, Norrakiah AS. Pesticide and antibiotic residues in freshwater aquaculture fish: chemical risk assessment from farm to table. Asian J Agro-Industry. 2010;3(3):328–34.
26. Islam. Chromosomal Antibiotic Resistance Mechanisms in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. Stockholm: Published by Karolinska institutet. University USAB; 2008.
27. Jayasinghe CVL, Ahmed SBN, Kariyawasam MGIU. The isolation and identification of *Vibrio* species in marine shrimps of Sri Lanka. J Food Agric. 2008;1(1):36–44.
28. Jeanette WPT, Antonio S, Chitlaa P. Novel β-lactamase genes from two environmental isolates of *Vibrio harveyi*. Antimicrob Agents Chemother. 2000;44(5):1309–14.
29. Jun L, Woo N. Pathogenicity of vibriosis in fish: an overview. J Ocean Univ China. 2003;2:117–28.

30. Kang CH, Shin Y, Kim W, Kim Y, Song K, Oh EG, Kim S, Yu H, So JS. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from oysters in Korea. Environ Sci Pollut Res Int. 2016;23:918–26.
31. Krumperman PH. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl Environ Microbiol. 1983;46(1):15–7.
32. Kumar PA, Patterson J, Karpagam P. Multiple antibiotic resistance profiles of *Vibrio cholera* non-01 and non-0139. Japan J Infect Dis. 2009;62:230–2.
33. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4.
34. Lee SE, Kim SY, Kim CM, Kim M, Kim YR, Jeong K, Ryu H, Lee YS, Chung SS, Choy HE, Rhee JH. The *pyrH* gene of *Vibrio vulnificus* is an essential in vivo survival factor. Infect Immun. 2007;75(6):2795–801.
35. Lesmana JM, Subekti D, Simanjuntak CH, Tjaniadi P, Campbell JR, Oyofo BA. *Vibrio parahaemolyticus* associated with cholera-like diarrhea among patients in North Jakarta. Indonesia Diagn Microbiol Infect Dis. 2001;39:71–5.
36. Letchumanan V, Yin WF, Lee LH, Chan KG. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. Front Microbiol. 2015a;33(6):1–11.
37. Letchumanan V, Chan K, Lee LH. An insight of traditional plasmid curing in *Vibrio* species. Front Microbiol. 2015b;6:735.
38. Li J, Zhou L, Woo NYS. Invasion route and pathogenic mechanisms of *Vibrio alginolyticus* to Silver Sea bream *Sparus sarba*. J Aquat Anim Health. 2011; 15(4):302–13.
39. Liu X, Wang D, Wang H, Feng E, Zhu L. Curing of plasmid pXO1 from *Bacillus anthracis* using plasmid incompatibility. PLoS. 2012;7:e29875.
40. Madigan MT, Martinko JM, Parker J. Brock biology of microorganisms. New York: Pearson Education, Inc.; 2003.
41. Manjusha S, Sarita GB. Characterization of plasmids from multiple antibiotic resistance vibrios isolated from molluscan and crustacean of Kerala. Int Food Res J. 2013;20(1):77–86.
42. Molina-Aja A, Garcia-Gasca A, Abreu-Grobois A, Bolan-Mejia C, Roque A, Gomez-Gil B. Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp. FEMS Microbiol Lett. 2002;213(1):7–12.
43. Nei M, Kumar S. Molecular evolution and Phylogenetics. New York: Oxford University Press; 2000.
44. Noorlis A, Ghazali FM, Cheah YK, Tuan-Zainazor TC, Wong WC, Tunung R, Pui CF, Nishibuchi M, Nakaguchi Y, Son R. Antibiotic resistance and biosafety of *Vibrio cholerae* and *Vibrio parahaemolyticus* from freshwater fish at retail level. Int Food Res J. 2011;18(4):1523–30.
45. Nsofor CA, Anyanwu NC, Ogbulie TE. High antibiotic resistance pattern observed in bacterial isolates from a tertiary Hospital in South East Nigeria. Int J Res Pharm Biosci. 2016;3(1):1–6.
46. Oramadike C, Ogunbanwo ST. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from seafoods in Lagos lagoon Nigeria. Cogent Food Agriculture. 2015;1:1041349.
47. Ottaviani D, Leoni F, Talevi G, Masini L, Santarelli S, Rocchegiani E, Susini F, Montagna C, Monno R, D'Annibale L, Manso E, Oliva M, Pazzani C. Extensive investigation of antimicrobial resistance in *Vibrio parahaemolyticus* from shellfish and clinical sources. Italy Int J Antimicrob Agents. 2013;42:191–3.
48. Pascual J, Macián MC, Arahal DR, Garay E, Pujalte MJ. Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, recA, pyrH, rpoD, gyrB, rctB and toxR genes. Int J Syst Evol Microbiol. 2010; 60(1):154–65.
49. Ramesh S, Manivasagan P, Ashokkumar S, Rajaram G, Mayavu P. Plasmid profiling and multiple antibiotic resistance of heterotrophic Bacteria isolated from Muthupettai mangrove environment, southeast coast of India. Curr Res Bacteriol. 2010;3:227–37.
50. Reboucas RH, Viana de Sousa O, Sousa Lima A, Roger FV, Carvalho PB, Fernandes RHV. Antimicrobial resistance profile of *Vibrio* species isolated from marine shrimp farming environments (*Litopenaeus vannamei*) at Cear'a, Brazil. Environ. Res. 2011;111:21–4.
51. Sahilah A, Laila R, Sallehuddin HM, Osman H, Aminah A, Azuhairi AA. Antibiotic resistance and molecular typing among cockle (*Anadara granosa*) strains of *Vibrio parahaemolyticus* by polymerase chain reaction (PCR)-based analysis. World J Microb Biot. 2014;30:649–59.
52. Saifedden G, Farinazleen G, Nor-Khaizura A, Kayali AY, Nakaguchi Y, Nishibuchi M, Son R. Antibiotic susceptibility profile *of Vibrio parahaemolyticus* isolated from shrimp in Selangor, Malaysia. Int Food Res J. 2016;23(6):2732–6.
53. Sanchez-Romero MA, Casadesus J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. PNAS. 2014;111(1):355–60.
54. Sawabe T, Kita-Tsukamoto K, Thompson FL. Inferring the evolutionary history of Vibrios by means of multilocus sequence analysis. J Bacteriol. 2007;189(21):7932–6.
55. Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AKMR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T. Updating the Vibrio clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius sp. nov*. Front Microbiol. 2013;4(414):1–14.
56. Scarano C, Spanu C, Ziino G, Pedonese F, Dalmasso A, Spanu V, Virdis S, De Santis EP. Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. New Microbiol. 2014;37(3):329–37.
57. Serrano, P.H. Responsible use of antibiotics in aquaculture, FAO fisheries technical paper no 469. Rome: Food and Agriculture Organization of the United Nations. 2005. http://www.fao.org/3/a-a0282e.pdf. Accessed 12 Oct 2018.
58. Spengler G, Molnar A, Schelz Z, Amaral L, Sharples D, Molnar J. The mechanism of plasmid curing in Bacteria. Curr Drug Targets. 2006;7:823–41.
59. Tall A, Hervio-Heath D, Teillon. A, Boisset-Helbert C, Delesmont R, Bodilis J, Touron-Bodilis A. Diversity of *Vibrio* spp. isolated at ambient environmental temperature in the Eastern English Channel as determined by pyrH sequencing. J Appl Microbiol. 2013;114:1713–24.
60. Talpur AD, Ikhwanuddin M. Dietary effects of garlic (Allium sativum) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch). Aquaculture. 2012;364-365:6–12.
61. Tan CW, Malcolm TTH, Kuan CH, Thung TY, Chang WS, Loo YY, Premarathne JMKJK, Ramzi OB, Norshafawatie MFS, Yusralimuna N, Rukayadi Y, Nakaguchi Y, Nishibuchi M, Radu S. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from short mackerels (*Rastrelliger brachysoma*) in Malaysia. Front Microbiol. 2017;8:1087.
62. Tanil GB, Radu S, Nishibuchi M, Rahim RA, Napis S, Maurice L, Gunsalam JW. Characterization of *Vibrio parahaemolyticus* isolated from coastal seawater in peninsular Malaysia. *Southeast Asian J*. Trop Med Pub Health. 2005;36(4): 940–5.
63. Thompson CC, Thompson FL, Vicente ACP. Identification of Vibrio cholerae and Vibrio mimicus by multilocus sequence analysis (MLSA). Int J Syst Evol Microbiol. 2008;58:617–21.
64. Vaseeharan B, Ramasamy P, Murugan T, Chen JC. In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. Int J Antimicrob Agents. 2005;26:285–91.
65. Yang Y, Xie J, Li H, Tan S, Chen Y, Yu H. Prevalence, Antibiotic Susceptibility and Diversity of *Vibrio parahaemolyticus* Isolates in Seafood from South China. Front Microbiol. 2017;8:2566.
66. Yano Y, Hamano K, Satomi M, Tsutsui I, Ban M, Aue-Umneoy D. Prevalence and antimicrobial susceptibility of *Vibrio* species related to food safety isolated from shrimp cultured at inland ponds in Thailand. Food Control. 2014;38:30–6.
67. Zanetti S, Spanu T, Deriu A, Romano L, Sechi LA, Fadda G. *In vitro* susceptibility of *Vibrio* spp. isolated from the environment. Int J Antimicrob Agents. 2001;17(5):407–9.
68. Zulkifli Y, Alitheen N. B., Raha, A. R., Yeap, S.K., Marlina, Son, R. and Nishibuchi, M. antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. Int Food Res J. 2009;16:53–8.

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