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. mytii* (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 para-haemolyticus, 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.

### 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      | 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%)                                                             |

**Antimicrobial susceptibility profile**

Antimicrobial susceptibility profile of the 380 *Vibrio* strains revealed 369 (97%) 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
isolates were susceptible to ampicillin and penicillin G.

\textbf{Antimicrobial susceptibility profile of the Vibrio spp.}

The antimicrobial susceptibility profiles of the 13 identified species of Vibrio are 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.

\textbf{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.

\textbf{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
(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%)
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...
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 one antibiotic.

![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](image)

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

| *Vibrio* species      | No of isolates | AMP | P   | B   | E   | S   | TE  | VA |
|-----------------------|----------------|-----|-----|-----|-----|-----|-----|----|
| *V. communis*         | 106            | 100 | 5   | 1   | 103 | 2   | 1   | 60 |
| *V. parahaemolyticus* | 95             | 73  | 7   | 15  | 80  | 7   | 8   | 36 |
| *V. alginolyticus*    | 70             | 55  | 7   | 8   | 43  | 19  | 8   | 17 |
| *V. vulnificus*       | 52             | 33  | 8   | 11  | 33  | 13  | 6   | 29 |
| *V. rotiferianus*     | 11             | 3   | 0   | 8   | 3   | 4   | 4   | 4  |
| *V. campbellii*       | 9              | 5   | 0   | 4   | 5   | 0   | 4   | 6  |
| *V. mytii*            | 8              | 8   | 0   | 0   | 8   | 0   | 0   | 8  |
| *V. furnissii*        | 7              | 5   | 2   | 0   | 7   | 0   | 0   | 3  |
| *V. harveyi*          | 5              | 4   | 1   | 0   | 4   | 1   | 0   | 2  |
| *V. tubiashii*        | 4              | 4   | 0   | 0   | 4   | 0   | 0   | 1  |
| *V. diabolicus*       | 1              | 1   | 0   | 0   | 1   | 0   | 0   | 1  |
| *V. fluvialis*        | 1              | 1   | 0   | 0   | 1   | 0   | 0   | 1  |
| Other *Vibrio* sp.    | 11             | 11  | 0   | 0   | 11  | 0   | 4   | 6  |

*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 | No of plasmid | After plasmid curing | 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* | PKS5 | 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/ 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, PJ5S | 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* | LLS, 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          |
| Species               | Strain number | Before plasmid curing Antibiotic profiles | No of plasmid | After plasmid curing Antibiotic profiles | No of plasmid |
|-----------------------|---------------|------------------------------------------|---------------|------------------------------------------|---------------|
| V. communis           | BS2           | Amp/ P/ VA                                | 1             | Amp/ VA                                  | Lost          |
| V. communis           | PK54, 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   | PKGI24        | Amp/ P/ B/ E/ VA                          | 1             | Amp/ P                                   | Lost          |
| V. parahaemolyticus   | NK6           | Amp/ P/ 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          |
three or more antibiotics. These findings were similar with a previous study that reported 68% of the Vibrio isolates were resistant 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

### 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 | Antibiotic profiles | After plasmid curing | No of plasmid |
|---------------|---------------|-----------------------|---------------|---------------------|----------------------|---------------|
| V. vulnificus | TL5, TS6      | Amp/ P/ E/ S/ VA      | 1             | No resistance       | Lost                 |               |
| V. vulnificus | PK8           | Amp/ P/ B/ S          | 1             | Amp/ P              | Lost                 |               |
| V. vulnificus | PJS16         | Amp/ P/ B/ S          | 1             | Amp/ P              | Lost                 |               |
| V. vulnificus | NL15          | Amp/ P/ B/ TE/ VA     | 1             | Amp/ P              | 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                 | Loss                 |               |
| 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       | Loss                 |               |
| Vibrio spp.   | LL18          | Amp/ P/ B/ E/ VA      | 1             | Amp/ P              | Lost                 |               |
| Vibrio spp.   | PKL21         | Amp/ P/ B/ S/ VA      | 1             | No resistance       | Loss                 |               |

AMP ampicillin, P penicillin G, B bacitracin, E erythromycin, S streptomycin, TE tetracycline, VA vancomycin
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. sullus) 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. Alternate steps between TCBS and TSB containing 1.5% normal saline were performed until a pure colony of Vibrio was obtained. 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′33.97″N, 100°26′36.96″E |
| 3 Ain Aquaculture Sdn. Bhd.   | Kota Bharu, Kelantan   | 6°7′50.80″N, 102°14′18.96″E |
| 4 Perniagaan Johari           | Besut, Terengganu       | 5°34′15.85″E, 102°31′18.79″E |
| 5 Aqua Hub Sdn. Bhd.          | Kuala Gula, Perak      | 4°59′51.20″N, 100°24′18.03″E |
| 6 KS Aquaculture Sdn. Bhd.    | Pulau Ketam, Selangor  | 3°29′34.8″N, 101°43′4.79″E |
| 7 Oasis Long Diann Bio-Tech Sdn. Bhd. | Banting, Selangor | 2°49′12.21″N, 101°30′56.40″E |
| 8 Aqua Genesis Sdn. Bhd.      | Port Dickson, Negeri Sembilan | 2°32′13.84″N, 101°48′21.6″E |
| 9 Smart Objectives Sdn. Bhd.  | Kukup Laut, Johor     | 1°25′22.8″N, 103°26′32.99″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-galactosidase (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 MgCl₂, 200 μM 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 1×10⁸ 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 acidine 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: Acidine 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; TSB: Tryptone soy broth; TSI: Triple sugar iron; VA: Vancomycin

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**Authors’ contributions**

DZ, MNAA, MTY 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.
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.

Consent for publication
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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