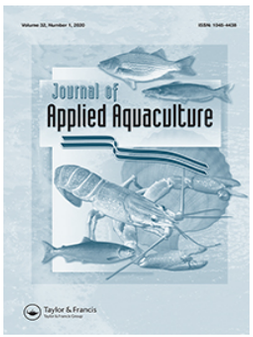

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Occurrence of *Vibrio cholerae* and *Vibrio parahaemolyticus* among milkfish farms in Zanzibar

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ABSTRACT

Fishing is among the main economic activities of the people of Zanzibar. Few fish dealers are transforming this sector into mariculture. Among the farmed fish is milkfish. Diseases are among the limiting factors in the development of the mariculture industry. Among other zoonotic diseases, vibriosis is caused by bacteria from the genus *Vibrio*. This study aimed to establish the occurrence of *Vibrio cholerae* and *Vibrio parahaemolyticus* among milkfish farms in Zanzibar. A total of 380 milkfish were sampled. Swabs were collected from gills, intestine, and kidney of each sampled milkfish. Preliminary identification of *V. cholerae* and *V. parahaemolyticus* was done by biochemical tests. PCR was run on 16S rRNA, outer membrane protein W, and collagenase genes to confirm *Vibrio* species, *V. cholerae*, and *V. parahaemolyticus* respectively. Almost one-third (32.1%) of all sampled milkfish were found to contain targeted *Vibrio*; 18% and 29.5% of the sampled milkfish were positive for *V. cholerae* and *V. parahaemolyticus* respectively.

KEYWORDS

Milkfish; prevalence; *Vibrio Cholerae*; *Vibrio parahaemolyticus*; Zanzibar

Introduction

Aquatic organisms are good pathogen vectors, and some of the pathogens they host infect them. Regardless of aquaculture being the fastest-growing food-producing industry in the world (Nadarajah and Flaaten 2017), it is challenged by many factors, including bacterial infections. These infections lead to fish loss and occasionally become a limiting factor in fish production (Bradford 2017).

Vibrio is one among many bacteria that infect fish (Amaro et al. 2015) and at the same time utilize fish as a bridge to infect fish consumers (Austin 2010). The genus *Vibrio* under the family of *Vibrionaceae* are gram-negative; non-spore-forming; and motile, straight, or curved rod-shaped (0.5 μm–1 μm) bacteria and indigenous to coastal marine systems (Nair et al. 2007: Pruzzo, Huq, and Colwell 2005). Most *Vibrio* are oxidase-positive and facultative anaerobes (Boyd 2007; Percival et al. 2013).

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Vibrio cholerae, V. vulnificus, V. anguillarum, and *V. parahaemolyticus* are the important pathogenic species in this genus (Silvester et al. 2017b). *V. anguillarum* and *V. parahaemolyticus* are known to cause vibriosis in fish (Bullock 1977), and *V. parahaemolyticus, V. cholerae*, and *V. vulnificus* are the most common causes of gastroenteritis, wound infections, and septicaemia in humans (Nair et al. 2007).

Diseases pose a substantial impact in the fish farming industry as they reduce the scale of cultured fish production. According to research reports done on milkfish farming in Zanzibar, fish diseases and mass mortality are said to be among the challenges facing the economic development of milkfish farming (Sullivan, Mmochi, and Crawford 2007). *Vibrio* is thought to be one of the diseases affecting milkfish farming in Zanzibar. The status of milkfish farming in respect to occurrence of *Vibrio* and other potential pathogens has not been determined in many developing countries, including Tanzania. This lack of information limits the efforts aimed at devising appropriate measures to prevent the occurrence of aquaculture-associated horrible diseases.

The use of marine water in milkfish farming in Zanzibar creates favorable environmental conditions for the proliferation and occurrence of vibriosis in fish (Davies et al. 1995). The current study therefore was aimed at determining the occurrence of *Vibrio cholera* and *Vibrio parahaemolyticus* in farmed milkfish in Zanzibar. The results of this study provide information on the occurrence of vibrio and can therefore be the basis for further research and formulation of control strategies for both fisheries and public health authorities.

Materials and methods

Study areas

This study was carried out in Zanzibar, on Unguja and Pemba Islands. Unguja Island had nine finfish farms, out of which only four farms are engaged in milkfish farming, with a total of 17 milkfish ponds. Pemba Island has 54 finfish farms, of which 41 are engaged in milkfish farming with 109 milkfish ponds. Samples were collected once every week from 12 January to 24 March 2018. Laboratory work was conducted in the Department of Veterinary Medicine and Public Health laboratories, College of Veterinary Medicine and Biomedical Sciences, SUA.

Study design and sample size determination

This study adopted a cross-sectional design. Due to unknown stocking density in most milkfish ponds, a simple random sampling technique was applied. The sample size was determined by a formula described by Thrusfield (1997): $N = Z^2 \{P(1\text{-}P)/E^2\}$, where *N* is sample size, *Z* is constant

(1.96), P is prevalence, and E is error margin (0.05). Based on a previous study conducted in India (Sudha et al. 2012), a known prevalence of *Vibrio* species in finfish from a tropical marine environment (Cochin) is 45.1%. The calculated sample size was 380. Therefore, 380 milkfish were sampled, 122 from Unguja and 258 from Pemba Island. The sample difference between the two islands was due to the difference in availability of fish and the number of milkfish farms.

Sampling procedure

In total, 24 farms were involved in this study. These included all the farms (n = 4) in Unguja Island and 20 farms randomly selected from 41 milkfish farms in Pemba Island. At the farm level, ponds were selected randomly. Twelve milkfish ponds were selected out of 17 in Unguja Island, and 40 milkfish ponds were selected out of 109 in Pemba Island. In total, 52 milkfish ponds were involved in this study. A seine net was used to fish out milkfish from each selected pond. Five to 20 milkfish were randomly selected from those fished out depending on availability and stocking density. A total of 380 milkfish were sampled.

Data collection

Each milkfish was screened for clinical signs or any pathological changes on the skin that could suggest bacterial infections. Gill swabs were taken before dissection, followed by gastrointestinal tract swabbing and kidney swabbing. This was done based on guidelines given by Sudha et al. (2012). Three swab samples were collected from each fish, making 1,140 swab samples. Collected specimens were immediately inoculated in 1.5 ml of Cary Blair transport media (Liofilchem s.r.l, Roseto, Italy), well labeled, and immediately preserved in a cool box and transported to Sokoine University of Agriculture Public Health Laboratory for other procedures within 48 hours. A cold chain was maintained throughout transportation.

Detection of occurrence of Vibrio organisms

Detection of *Vibrio* followed standards described in the *Bacteriological Analysis Manual for Vibrio* (Kaysner and DePaola 2004). Sample enrichment for isolation of *Vibrio* was done using Alkaline saline peptone water (APW), which is a selective enrichment broth favoring halophile microorganisms only (Robert 2012). Following procedures described previously (Di et al. 2015), each swab sample was enriched in 10 ml of alkaline saline peptone water (Himedia, Mumbai, India) and incubated for 6 h at 37°C. One ml of

the solution was then transferred into 10 ml of fresh alkaline saline peptone water and incubated at 37°C for 18 hours.

A drop from the supernatant layer was inoculated on Thiosulfate Citrate Bile Sucrose (TCBS) agar (Himedia, Mumbai, India) as a selective agar for *Vibrio cholerae* and *Vibrio parahaemolyticus* and incubated at 37°C for 24 hours. Yellow mucoid mushroom-top-shaped colonies of 3–5 mm diameter, presumptive *V. cholerae* (Singh, Chaturvedi, and Bagchi 2017), and flat bluegreen 3–4 mm diameter colonies, presumptive of *V. parahaemolyticus* (Cai, Han, and Wang 2006), were picked out from the massive growth and subcultured on the same agar at the same conditions to clearly show the morphology of their single colonies. Isolated colonies were then streaked on Mueller Hinton ager (Himedia, Mumbai, India) and incubated at 37°C for 24 hours for purification. Although TCBS is a medium useful for isolation of *V. cholerae* and *V. parahaemolyticus*, selectivity of the medium is such that it may not always suppress growth of other organisms, such as *Proteus spp., Aeromonas spp*., or *Staphylococcus spp*. Additional tests were found necessary (Gopal et al. 2005).

Vibrios are known to be gram-negative bacteria, rod-shaped with flagella. Peptone water was used to make smears of pure colonies on sterile glass slides, and gram staining was done. Dried slides were viewed under a microscope with x100 magnification. The gram-negative, slightly curved rod-shaped organisms were identified, and their colonies were tested further.

Triple Sugar Iron (TSI) agar (Himedia, Mumbai India) of a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous ammonium sulfate, was prepared. Isolated gram-negative colonies were inoculated in the slant and incubated for 24 hours at 37°C. Samples that produced acidic slant over acidic butt (A/A) that is yellow over yellow with no gas production were presumptive of *V. cholerae*. Samples with alkaline slant over acidic butt (K/A) that is red over yellow with no gas production were presumptive of *V. parahaemolyticus* (Silvester et al., 2017a). These were preserved for further testing.

Oxidase reagent discs (Himedia, Mumbai India) were used to test oxidation of the isolated bacteria. All Vibrios are known to be oxidase positive, turning to blue-purple within 5 seconds of testing (Mac Faddin, 1976). All positive colonies were preserved for molecular confirmation.

Storage of isolated colonies

Isolated colonies were stored in nutrient broth containing 30% glycerol and preserved in a deep freezer (−80°C). Copies of the same were also stored in Mueller Hinton agar (Himedia, Mumbai India) plates and kept in a refrigerator (0–4°C).

Confirmation of the isolates by polymerase chain reaction

After purification in TSA+2%NaCl agar at 37°C for 24 hours, colonies were harvested into 200 µl double-distilled water and boiled at 100°C for 10 min. Cell debris were removed by centrifugation at 12,000 rpm for 3 min, and the supernatant containing template DNA was taken into a fresh microfuge tube for PCR assay (Robert-Pillot et al. 2014).

According to procedures recorded by Kumar et al. (2011) and Izumiya et al. (2011), Uniplex PCR was run to detect *Vibrio* Genus-specific 16S-rRNA gene (primers as shown on Table 4), then followed by Multiplex PCR for the positive samples to detect species-specific genes for *V. cholerae* and *V. parahaemolyticus* (primers and target genes shown on Table 4). EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan) was used for both reactions. DNA concentrations of each sample were adjusted to 50 ng/µl for PCR. Uniplex PCR mixture contained 2.0 µM of primer, 4.0 µM of template DNA, 7.0 µM of DI water, and 12.0 µM master mix, making 25 µM mixture. Multiplex PCR mixture contained 1.0 µM of each primer, 4.0 µM of template DNA, 7.0 µM of DI water, and 12.0 µM master mix making 25 µM mixture. The mixtures then followed by 15 minutes of initial denaturation at 93°C; 35 cycles of denaturation at 92°C for 40 seconds, annealing at 57°C for 1 minute, and extension at 72°C for 1.5 minutes, and a final extension at 72°C for 7 minutes. PCR products were then stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO) and visualized in 1.0% agarose gels (Sigma-Aldrich) using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA). Electrophoresis was run at 100 volts for 40 minutes, and the amplicons showed clear bands, as in Figures 1 and 2. The confirmed isolates were stored at – 80°C.

Data analysis

The data were compiled, cleaned, and stored in Microsoft Excel. The data were then analyzed by SPSS software version 16. Descriptive statistics, particularly frequencies, were computed to determine proportions of positive fish by ponds and farms. A chi-square test was used to test the significance of differences in proportions at critical probability of $P < .05$ and 95% confidence interval. Using 2 × 2 contingency tables, the strength of associations between variables (pond condition and managerial practices) against fishpond positivity for *V. cholerae* and *V. parahaemolyticus* was determined.

Results

Occurrence of Vibrio

A total of 380 gill swabs, 380 intestinal swabs, and 380 kidney swabs were collected from 380 fish. On primary culture, 68.4% of the total sampled fish

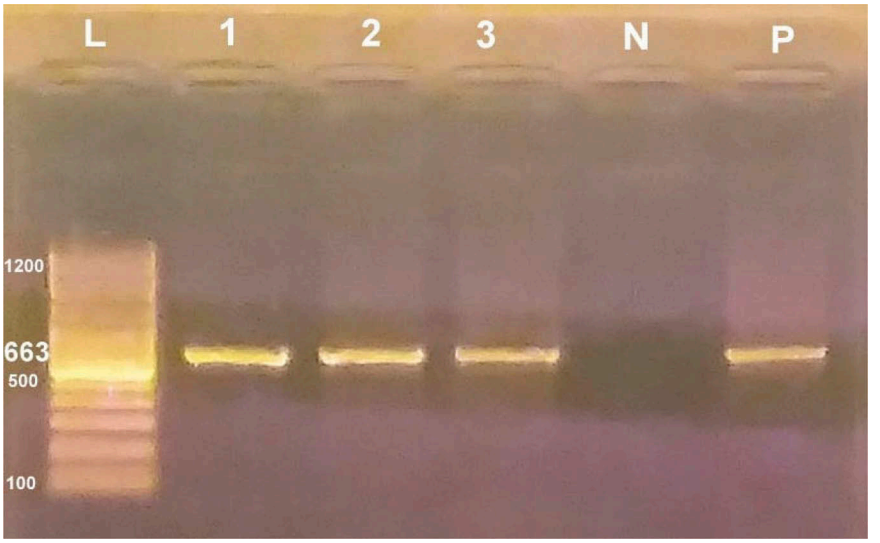

Figure 1. Agarose gel showing 663 bp m-PCR amplification products for detection of *Vibrio* species. Lane L = 100 bp DNA ladder; Lane 1 = U1B3KP; Lane 2 = P14A3IP; Lane 3 = P7A3IP, Lane N = Negative control (water) and Lane *P* = Positive control (*V. cholerae* from bank SUA).

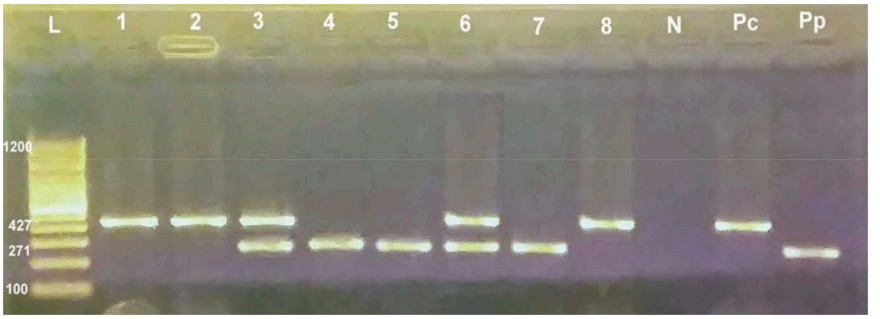

Figure 2. Agarose gel showing 427 bp m-PCR amplification products for detection of *Vibrio cholerae* and 271 bp m-PCR amplification products for detection of *Vibrio parahaemolyticus*. Lane L = 100 bp DNA ladder; Lane 1 = U1B3GC; Lane 2 = P13A2GC; Lane 3 = U2A3GC; Lane 4 = P6B3KP; Lane 5 = P14A3IP; Lane 6 = P7A3IP; Lane 7 = P9A4KP; Lane 8 = P14A2GC; Lane N = Negative control (watera); Lane Pc = Positive control (*V. cholerae* from bank SUA) and Lane *P* = Positive control (*V. parahaemolyticus* from bank SUA).

yielded organisms suggestive of targeted *Vibrio* species, 44.1% of which were suggestive of *V. cholerae* and 48.5% were suggestive of *V. parahaemolyticus*. The rest were suggestive of both *V. cholerae* and *V. parahaemolyticus*. Isolated organisms suggestive of *Vibrio* were subjected to gram staining, and 30.2% were gram-negative short curved rods. A TSI test was thereafter done, and 94.0% isolates were suggestive of *V. cholerae* and *V. parahaemolyticus* at 40.0% and 60.0% respectively. This was followed by a oxidase test: 98.3% isolates were oxidase positive. Final isolates confirmed by PCR molecular techniques and all

presumptively *V. parahaemolyticus* were positive, and 98% of presumptive *V. cholerae* were positive. It was found that 32.1% of sampled fish were positive for the selected *Vibrio* species in the proportion 2:1 *V. cholerae* and *V. parahaemolyticus* respectively. A majority of infested fish (80.6%) originated from Pemba Island; the rest were from Unguja Island. At the species level, the overall prevalence was 17.9% and 29.5% for *V cholerae* and *V. parahaemolyticus* respectively. Electrophoresis results and amplicons gave clear bands, as in Figure 1, showing strains U1B3KP, which originates from Unguja Island, and P14A3IP and P7A3IP from Pemba Island. Figure 2 shows amplification of strains U1B3GC and U2A3GC from Unguja Island and P13A2GC, P6B3KP, P14A3IP, P7A3IP, P9A4KP, and P14A2GC from Pemba Island. Water was used as the negative control, and *V. cholerae* and *V. parahaemolyticus* were taken from the SUA bacteria bank.

Proportion of positive farms

The majority of farms involved in this study (85%) had at least one isolate; 91.7% contained *V. parahaemolyticus* and 75.0% *V. cholerae*. A significant proportion (66.7%) of the farms had both *V. parahaemolyticus* and *V. cholerae*.

Occurrence of Vibrio at the farm level

Sampled fish from some farms did not have any positive isolate. Table 1 shows the number of fish containing *Vibrio* at the farm level.

Occurrence of Vibrio at the pond level

Most (73.0%) of sampled milkfish ponds contained *Vibrio*: 58.3% contained both *V. cholerae* and *V. parahaemolyticus*, and the rest contained either *V. Parahaemolyticus* (33.3%) or *V. cholerae* (8.3%) only.

Proportion of positive swabs and distribution of selected Vibrio species at organ level

Of the swabs, 6.7% of positive gill swabs, 4.4% of positive intestine swabs, and 2.2% of kidney swabs had both *V. cholerae* and *V. parahaemolyticus* isolates. Tables 2 and 3 shows occurrence of *Vibrio* by organs sampled from milkfish

Table 1. Showing occurrence of vibrios at farm level in milkfish farms of Zanzibar.

	Minimum occurrence	Maximum occurrence	Median	Mode	Mean	Std Dev
Occurrence of vibrios	0.00	9.0	5.0	1.0	4.2	3.4
V. cholerae	0.0	6.0	2.5	0.0	2.5	2.1
V. parahaemolyticus	0.0	4.0	2.0	0.0	1.6	1.4

Table 2. Occurrence of vibrios by organs sampled from milkfish in Zanzibar.

	Total sampled	Proportion	*V. cholerae*	*V. parahaemolyticus*
Gills	380	17.0%	8.1%	8.9%
Intestine	380	17.9%	7.2%	10.7%
Kidney	380	9.8%	3.6%	6.2%

Table 3. Distribution of isolates in fish by organs.

	Proportion of infested fish	*V. Cholerae*	*V. Parahaemolyticus*
Fish with all organs positive	4.0%	2.0%	2.0%
Positive fish in gills only	20.0%	10.0%	10.0%
Positive fish in intestine only	22.0%	11.99%	10.01%
Positive fish in kidney only	14.0%	6.0%	8.0%
Positive fish in gills and intestine	10.0%	6.0%	4.0%
Positive fish in intestine and kidney	4.0%	0.72%	3.28%
Positive fish in gills and kidney	2.0%	0.0%	2.0%

Table 4. Oligonucleotide primers to be used in this study.

Organism (Target gene)	Primer sequence (5′–3′)	Product size (bp)	Reference as cited by Kumar et al. (2011) and Izumiya et al. (2011)
All *Vibrio spp*., (16S rRNA)	F: CGGTGAAATGCGTAGAGAT R: TTACATGCGATTCCGAGTTC	663	(Tarr et al. 2007)
Vibrio cholera (outer membrane protein W)	F-CACCAAGAAGGTGACTTTATTGTG R-CGTTAGCAGCAAGTCCCCAT	427	(Evans, Piermarini, and Choe 2005)
V. parahaemolyticus (collagenase)	F-GAAAGTTGAACATCATCAGCACGA R-GGTCAGAATCAAACGCCG	271	(Tarr et al. 2007)

in Zanzibar and the distribution of isolates in sampled fish in respect to organs respectively.

Discussion

This study revealed a *Vibrio* prevalence of 32.1% in Zanzibar. This prevalence is higher compared to the 23.3% reported by Hounmanou (2015) in a study that was conducted in Morogoro on the Tanzania mainland (Hounmanou 2015). Nyambuli (2017) studied the occurrence of *V. cholerae* in sardines; a prevalence of 9.0% was found. In India, however, Sudha et al. (2012) found a higher *Vibrio* prevalence (45%) in shrimps. The difference in prevalence may be due to difference in location; the two mentioned studies done in Tanzania were carried out in inland water bodies dissimilar to this study, which was conducted in a marine environment. Findings of the current study corresponds to what was reported by Yang et al. (2008), who found the general prevalence of *Vibrio* species in cultured fish to be 19.4% in China. Sudha et al. (2012) also reported a *Vibrio* species prevalence of 45.1% in milkfish cultured in Cochin, India. Zanzibar Island has no seasonal variation between January and March. This study did not reveal a statistically significant difference between *Vibrio* species isolated from samples collected throughout the three months.

The clear difference in *Vibrio* species prevalence between Unguja and Pemba Islands is due to milkfish ponds in Pemba Island being situated in residential areas. In Unguja Island, all the studied milkfish farms are located in reserved areas away from social activities. Human behavior in Pemba Island attributed to lack of sanitation facilities and other conduct common to coastal communities (Oktari et al. 2015), which use the Indian Ocean as the dumping site. This could be one of the main reasons for these findings.

Distribution of selected *Vibrio* species among sampled organs is in agreement with Terentjeva et al. (2015), who said that *Vibrio* species can be found on the skin, chitinous shell, gills, intestinal tracts, kidney, and liver of fish or shellfish. This finding also corresponds with a study on the occurrence of a potentially pathogenic *Vibrio* species in sea foods obtained from Oron Creek, which revealed a prevalence of 17% of *V. parahaemolyticus* from fish gills (Adebayo-Tayo et al. 2011). *Vibrio* has also been isolated from fish kidney by Jayasinghe, Ahmed, and Kariyawasam (2010) in marine fish of Sri Lanka. In general, this study reports a prevalence of 17.9% and 29.5% for *V. cholerae* and *V. parahaemolyticus* respectively in farmed milkfish in Zanzibar.

Conclusion and recommendation

A good proportion of the farmed milkfish in Zanzibar were found to have *Vibrio cholerae* and *Vibrio parahaemolyticus*, though this study did not find out whether these are toxic strains. Since *Vibrio* bacteria are known to be zoonotic, their occurrence in farmed milkfish in this study calls for the institution of control measures to limit their persistence in farmed fish and their transmission to humans. Their transmission from fish to humans can be caused by improper handling during preparation and cooking. Furthermore, milkfish farmers should consider wearing protective gear during pond management practices to avoid wound infections with *V. parahaemolyticus*. To prevent the possible occurrence of the clinical form of *Vibriosis* in infested fish, proper fish farm management practices are insisted on. Further studies involving other fish and *Vibrio* species is recommended.

Disclosure statement

No potential conflict of interest was reported by the authors.

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